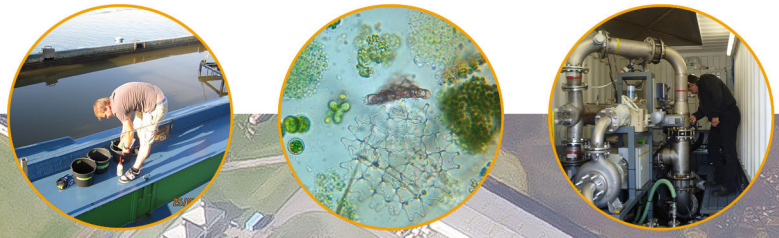
CATHELCO LTD
BALLAST WATER MANAGEMENT SYSTEM

PART 4 ANNEX 1d

NIOZ Report Land Based Tests

Author: NIOZ



The Cathelco UV ballast water treatment system: results of the 2012 land-based IMO G8 tests at NIOZ

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NIOZ Ballast Water Report 2013-2
NIOZ Royal Netherlands Institute for Sea Research

The Cathelco UV ballast water treatment system: results of the 2012 land-based IMO G8 tests at NIOZ

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Aerial photo on cover by Pieter de Vries Photography. All other photos by NIOZ.

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EXECUTIVE SUMMARY

The CATHELCO UV Ballast Water Treatment System (BWTS) of CATHELCO Ltd was tested for IMO type approval at the NIOZ Royal Netherlands Institute for Sea Research from April to October 2012. The CATHELCO BWTS is a modular ballast water treatment system with a maximum treatment rated capacity of 200m^3/h that is installed in bypass to the main ballast line. Treatment of ballast water is achieved through a two-step process. At intake the first step is filtration using a 40 µm mesh screen. In the second step filtered water is disinfected using two medium pressure UV lamps. At discharge the ballast water is treated a second time with UV radiation.

The CATHELCO BWTS was tested with marine and fresh water. For all tests the holding time before discharge was five days.

In general, the G8 requirements for the abiotic water quality and the abundance and biodiversity of organisms were met for both salinity ranges. Especially the biodiversity in the test water was extremely high. In addition, low UV-T values were encountered during freshwater testing.

Treatment with the CATHELCO UV system did not negatively change the abiotic quality of the discharge water in terms of environmental variables and disinfection by-products.

At both salinity regimes a more than sufficient reduction of organisms in the >50 µm size class generally led to compliance with the D-2-standard. The biological efficacies at both salinities for this size class surpassed the combined D2-G8 requirements.

In addition, the 10-50 µm size class organisms were sufficiently reduced at both salinity regimes which led to compliance with the D-2-standard. The biological efficacies at all salinities for this size class surpassed the combined D2-G8 requirements.

Of the indicator microbes the concentrations of *E. coli*, which were present in the freshwater tests, were sufficiently reduced which led to compliance with the D-2-standard.

In conclusion: the CATHELCO UV Ballast Water Treatment System as tested at NIOZ in 2012 is an environmentally safe ballast water management system with a high biological efficacy that generally meets and exceeds the D-2 Ballast Water Performance Standard.

SUMMARY TABLES CATHELCO UV WATER TREATMENT SYSTEM

Averages and ranges of organisms relevant for the G8-guidelines and the D-2 ballast water performance standard obtained in 5 (marine) and 6 (freshwater) passed tests.

Marine water	Test water (T0)		Control (T5)		Treated (T5)		
(5 tests)	average	min - max	average	min - max	average	min - max	unit
Organisms ≥ 50µm	111,000	109,000 - 113,000	28,000	17,000 - 41,000	4	1 - 9	per m3
Organisms 10≤µm≤50	0	970-1660	94	58-122	3	0-6	per mL
Heterotrophic bacteria	2.2	1.3 - 2.8	1.0	0.9-1.2	0.8	0.1 - 1.5	x1,000,000 per mL
E. coli	<10	<10	<10	<10	<10	<10	cfu/100mL
Enterococci	1	<1 - 2	<1	<1	<1	<1	cfu/100mL

Fresh water	Test water (T0)		Control (T5)		Treated (T5)		
(6 tests)	average	min - max	average	min - max	average	min - max	unit
Organisms ≥ 50µm	169,000	113,000-244,000	100,000	36,000 - 371,000	2	0 - 9	per m3
Organisms 10≤µm≤50	1870	1025-1875	172	81-381	5	2-7	per mL
Heterotrophic bacteria	1.9	1,8 - 2.0	0.9	0,6 - 1,3	1.4	0.7 - 2.0	x1,000,000 per mL
E. coli	1200	15 - 3400	300	<10 - 500	<10	<10	cfu/100mL
Enterococci	1	<1 - 2	<1	<1	<1	<1	cfu/100mL

Range of relevant environmental variables for which the treatment system was tested.

variable	range	unit
Salinity	0.4 - 36.1	g/kg (PSU)
DOC	2 - 7	mg/L
POC	5 - 36	mg/L
TSS	11 - 86	mg/L
Temperature	9 - 17	°C
UV-T	53 - >62	%
Organisms ≥ 50µm	109 - 244	x 1000 per m3
Organisms 10≤µm≤50	970 - 1875	per mL

ACKNOWLEDGEMENTS

The author thanks the members of the ballast water team: Jan Boon (quality manager), Eveline Garritsen, Josje Snoek, Alex Blin, Eva Immler, Dennis Mosk and Dörte Poszig for their contributions to the CATHELCO tests and this report. Jaap Witte (Klusbedrijf Jaap Witte, Den Burg) and André Smit (Smittech Texel, Den Hoorn) provided technical assistance at the harbour test facility. Ph.D. student Cees van Slooten contributed to the G8 tests with a novel compliance technique. The technical staff of the NIOZ planktonlab is acknowledged for general support.

1 INTRODUCTION

Ships transport five to ten billion tons of ballast water annually over the globe (Endresen et al. 2004). This ballast water is loaded with particulate sediment and an enormous variety of living organisms ranging from juvenile stages, larvae and eggs of fish and larger zooplankton (Williams et al. 1988, Carlton & Geller 1993) to macroalgae, phytoplankton (Hallegraeff et al. 1997, Hamer et al. 2000), bacteria and viruses (Gollasch et al. 1998). In general, these organisms belong to the natural ecosystem in and around the port of origin but they might not be occurring naturally in the coastal waters and port of destination at the end of a ship's voyage. This can have a high impact on the natural ecosystem and can cause significant ecological and economic damage (Hoagland et al. 2002), when it results in a decrease of stocks of commercially valuable fish and shellfish species. If no action is taken, the problem of invasive species may increase considerably for several reasons. Ships are getting larger, faster and the amount of traffic across the oceans is expected to increase rapidly in the coming decades. This results in an increased volume and transfer rate of ballast water and, therefore, also an increased chance of non-indigenous organisms to have large enough numbers for settling and expanding. Efforts to reduce pollution of ports and coastal waters have also improved the quality of the aquatic environment in these areas but this may increase the susceptibility to invasive organisms. The problem of invasive species is considered as one of the four major threats of the world's oceans next to land-based marine pollution, overexploitation of living marine resources, and physical alteration/destruction of habitats.

To minimize these risks in the future, the International Maritime Organization (IMO) of the United Nations has adopted the Ballast Water Management Convention (BWMC) in 2004 (IMO 2005). The Convention states that all ships (>50,000 in number) should install proper ballast water treatment (BWT) equipment on board between 2009 and 2016. Although at present the number of countries having ratified the Convention has reached the required minimum, the required tonnage has not been reached. Yet the expectation is that the Convention will be implemented in the near future.

As a temporary solution ships may reduce the risk of invasive species by performing ballast water exchange during their voyage when passing through deep water (>200 m depth and 200 NM from the coast). Ballast water exchange faces many problems as to feasibility, safety and efficacy. For a large part of the ships' voyages the required depth and/or distance to shore requirements are not met. Ballast water exchange can affect a ship's construction stability and in rough seas exchange is not possible because of the risk to ship and crew. Treatment of ballast water is therefore considered to be the best solution to reducing the risk of invasive species.

During recent years numerous solutions for treatment of ballast water have been mentioned and tested with the ultimate goal to reduce the amount of organisms in ballast water (Tsolaki & Diamadopoulos 2010, Goncalves & Gagnon 2012). However, apart from a high efficacy there is more needed for a BWT system to be a good system. Apart from being biologically effective, the system should be practicable, environmentally acceptable and also cost effective.

Role of NIOZ in ballast water research and certification of BWTS

NIOZ is the national oceanographic research institution of the Netherlands at which several methods have been developed to count and characterize different classes of planktonic organisms, including viability, and a large number of abiotic variables. This set of analytical methods also forms the core of the measurements required for the land-based tests which are part of the requirements for the certification of BWTS according to the IMO guidelines. The NIOZ head office on the island of Texel is located at the Marsdiep tidal inlet on the border of the western Wadden Sea and the coastal North Sea, which is a highly productive shallow sea area with a large variety of natural plankton. In addition, the nearby Lake IJssel is a rich source of planktonic organisms for freshwater tests. The main tests are carried out at the NIOZ harbour, while the analyses are done partly directly at the harbour and partly at

the well-equipped laboratories of the nearby institute. The ballast water research group is embedded in the scientific department of Biological Oceanography. Lloyd's Register confirmed that NIOZ has the required infrastructure, procedures, appropriate experience and qualified personal to undertake all the requirements as a land based testing facility for the testing and evaluation of ballast water management systems. More details follow in the next chapters. Besides the certification testing, the group is also involved in further method development for land-based and shipboard testing, as well as compliance monitoring and enforcement.

2 GENERAL DESCRIPTION

Figure 2.1. Aerial view of the NIOZ harbour (lower right), NIOZ and the TESO ferry connecting the island of Texel with the main land (top). The Mokbaai is the source for additional suspended solids.
©Photo: Simon Smit Photography, Den Burg, Texel.

2.1 NIOZ profile

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is an institute of the Netherlands Organization for Scientific Research (NWO). The institute employs about 340 people at locations on the island of Texel on the border of the North Sea and the Wadden Sea (main location) and in Yerseke in the southwest of the country. The annual budget is approximately €30 million.

The mission of NIOZ is to gain and spread scientific knowledge on seas and oceans for a better understanding and sustainable use of our planet, to manage the national facilities for sea research and to support marine research and education in the Netherlands, and in Europe.

In order to fulfil its mission, the institute performs tasks in four specific fields.

Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. Increasingly, the institute also carries out research based on societal issues. The senior scientists at NIOZ all participate in international research projects. Several of them also hold a professorship at Dutch or foreign universities.

Education: The institute educates PhD students and master students of universities and schools for professional education. Together with several universities, NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists are also appointed as professor at Dutch and foreign universities.

Marine Technology: NIOZ has its own workshops for mechanical, instrumental en electronic engineering. Here, marine research equipment is being designed and built according to the wishes of our individual scientists.

Facilities: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories and large research equipment. Our ocean-going research vessel 'Pelagia' is shared on a European level in the 'Ocean Facilities Exchange Group' (www.ofeg.org).

The basic scientific disciplines at NIOZ are physics, chemistry, biology and geology. Multidisciplinary sea research is regarded as one of the main strengths of the institute. Therefore, the research is organised in 5 multi-disciplinary themes: 'Open ocean processes, Sea floor dynamics, Wadden and shelf sea systems, Climate variability and the sea and Biodiversity and ecosystem functioning'.

Together with a number of oceanographic partners, NIOZ also maintains the popular marine website www.seaonscreen.org.

For more information, please contact our Communication & PR department at cpr@nioz.nl, or visit our website at www.nioz.nl.

NIOZ has extensive experience in the field of ballast water and the testing of ballast water treatment technologies at its harbour on the island of Texel. During the past seven years, several pilot tests for ballast water treatment have been conducted at the NIOZ harbour. Between 2007 and 2013, 10 full scale land-based tests have been carried out for Final and Type Approval of which 8 have received Type Approval so far.

2.2 North Sea Ballast Water Opportunity project

From 2009 onwards, the activities of NIOZ in ballast water research have been organized in a broader framework, the North Sea Ballast Water Opportunity project (www.NorthSeaBallast.eu). This project is an initiative of the BSH (Federal Maritime and Hydrography Agency, Germany) and NIOZ and involves all relevant stakeholders within the maritime sector in the North Sea region: governmental institutions, inter-governmental and non-governmental organisations, industry and scientific and technological institutes. This structure and participation offers a broad and sound base for the project in support of a successful implementation of the IMO Convention in the region. Moreover, the project being one of the largest and most integrative of its kind, the objectives (investments) will become available as a model for other European maritime regions as well as other regions across the globe. To facilitate this initiative, funding was received from the North Sea Interreg IVB (an ERDF program). For the embedding in a more global strategy the project is liaising with the Globallast II initiative of the IMO and currently involves also comparable research initiatives in the US (GSI, MERC and Golden Bear).

2.3 NIOZ test facility

2.3.1 Marine water: high and intermediate salinities

The land-based tests were carried out at the NIOZ harbour on the island of Texel from April to October 2012. Three coated tanks of 300 m³ each, situated on the so-called Pelagia quay, simulated a ship's ballast water tanks. The tanks were cleaned with high pressure steam after each test. Water samples can be taken from bypasses of the standard piping (DIN 200) used to fill and to empty the tanks (Figure 2.2). According to the requirements of the G8 Guidelines, sampling points are located directly behind the ballast water pump, and at the pipe directly behind the BWTS.

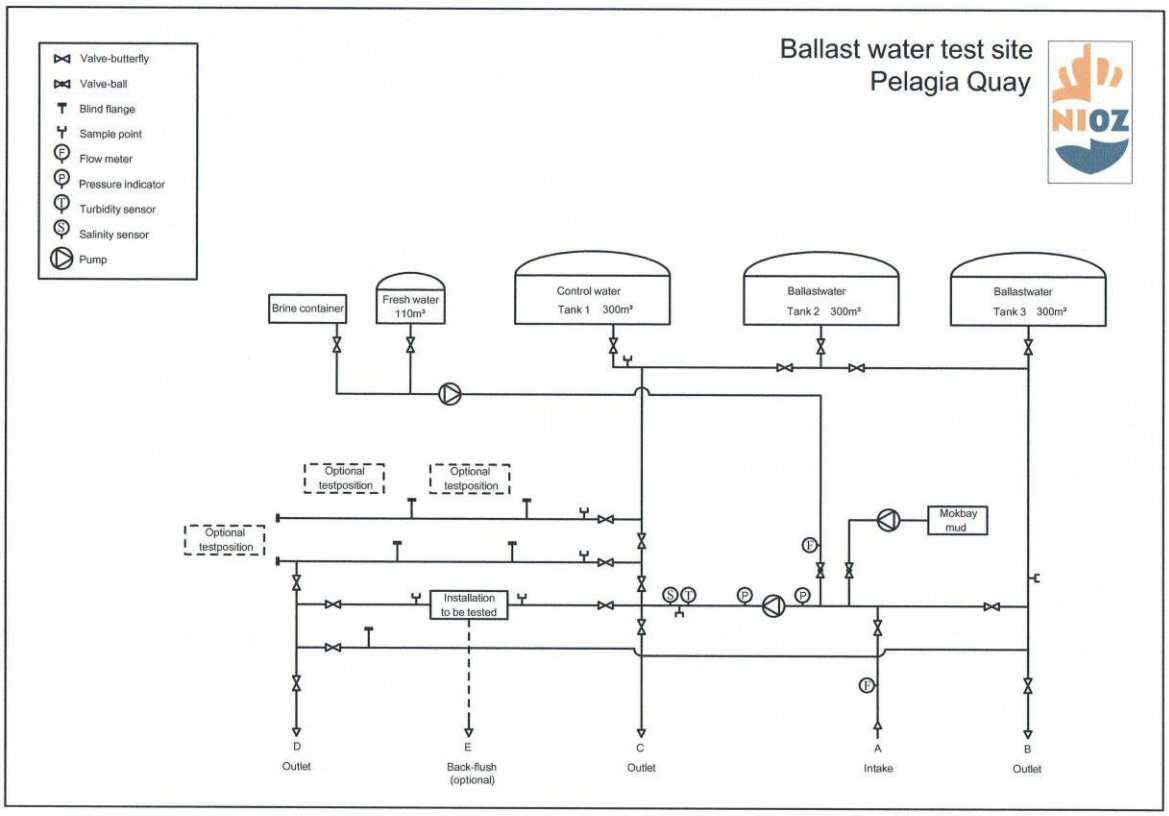

Figure 2.2. Piping and Instrumentation diagram of the Pelagia quay test site at the NIOZ harbour. The installation to be tested is a UV-treatment system. The installation consists of three ballast water tanks, one for control (untreated) water and two for treated water. Brine or freshwater can be added to adjust the salinity of the test water. Mud can be added to increase the concentration of Total Suspended Solids (TSS). Flow rates, system pressures, salinity and turbidity are monitored during intake and discharge.

The CATHELCO UV system was connected to a water pump (capacity of up to 250 m3/h) drawing in water from the NIOZ harbour. The harbour has direct access to the Wadden Sea and therefore the origin of the test water changes with the tide. Furthermore, provisions were made to allow the addition of brine water and/or fresh water in order to adjust the salinity of the natural water of the NIOZ harbour with ±2 PSU to the required test conditions of brackish water and marine water with a minimum of 10 PSU difference. A detailed description of the test installation is presented in Figure 2.2.

2.3.2 Fresh water

In 2012, NIOZ started performing freshwater tests using water from nearby Lake Ijssel (Figure 2.3) using a 650 m3 capacity ship. The intake water was pumped into the hold with tubes that were suspended at a depth of a maximum of 1 meter below the water surface. The freshwater test water was transported by ship over a relatively short distance across the Wadden Sea within 12 hours to the NIOZ harbour. The day following the intake, i.e. within 24 hours, the test water is used in the NIOZ test facility.

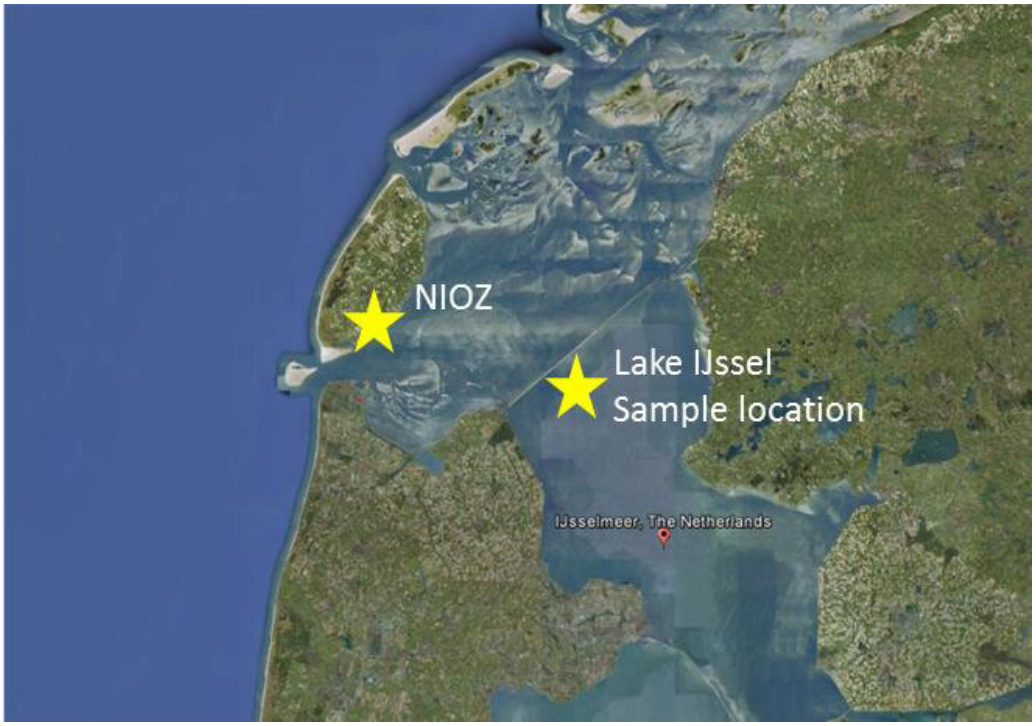

Figure 2.3. Fresh water sample location in nearby Lake IJssel. The fresh water is transported to NIOZ by boat within hours after sampling.

Figure 2.4. The submersible pump that is used to transfer fresh water from a ship to the NIOZ test facility.

An Amarex KRTK 100-401/354WG-S pump, suspended from a crane standing on the NIOZ Pelagia quay (Figure 2.4) was used to transfer the fresh water from the ship into the NIOZ installation. The crane adjusted the pump in relation to the freshwater surface level changing as a result of deballasting and tide. The pump was connected to the NIOZ installation (Figure 2.2) by a flexible hose. The fresh water was subsequently treated the same as the water in the marine water tests.

2.3.3. Test season: time planning

The intermediate and high salinity range test season at the NIOZ harbour is restricted to spring and summer. In this period of the year, sufficiently high numbers of organisms are naturally present in the North and Wadden Sea. At NIOZ the test water is not enriched with organisms, neither artificially cultured nor collected at sea. In general, early spring sea water has a lower salinity than sea water in summer due to a decrease in salinity as a result of higher river discharge. A decrease in wind speed during spring will lead to diminishing concentrations of total suspended solids (TSS) and, therefore, sediment from the nearby Mokbaai (Figure 2.1) is added to increase TSS to the required value of 50 mg/l for brackish water.

In February sampling and measurements of the harbour water start in order to monitor the start of the spring plankton bloom. In March the first G8 tests may be performed, depending on natural circumstances such as water temperature and underwater light climate that affect the plankton development.

The first set of tests is carried out at the intermediate salinity range of G8 (§2.3.17) because the freshwater content of the Wadden Sea is relatively high in early spring. Test water is pumped from the harbour at low tide when low salinity Wadden Sea water flows towards the North Sea. The second set of high salinity tests is performed in late spring or early summer. Test water is pumped from the harbour at high tide when relatively saline North Sea water flows towards the Wadden Sea.

After consultation with the BSH, the CATHELCO BWTS tests carried out in spring and early summer of 2012 were combined to one salinity (saline) range. This meant that an additional series of freshwater tests had to be performed. The first appropriate months for these freshwater tests were September and October.

2.4 Profile of CATHELCO Ltd.

CATHELCO Ltd was formed in 1956 and has become a world leading supplier of cathodic protection equipment to the shipping and offshore marine markets.
The parent company based in Chesterfield, United Kingdom manufactures marine pipe work anti-fouling (AF) and impressed current cathodic protection (ICCP) systems.
The CATHELCO Group has grown through a series of acquisitions beginning with Corrintec Ltd in 1995. Today, Corrintec Marine continues to operate as a wholly owned subsidiary serving the military sector worldwide.

In 2005, the company established CATHELCO Korea, a subsidiary engaged in manufacturing and distribution serving the Korean shipbuilding market.

More recently, in 2010, Seafresh Desalinators Ltd was acquired which specialises in reverse osmosis water makers from a manufacturing facility in Bournemouth, United Kingdom.
To serve the shipbuilding and repair industry in South East Asia, CATHELCO S.E.A. based in Singapore, became a subsidiary in 2011.

In addition to its marine engineering activities, the CATHELCO Group encompasses Casting Repairs Ltd, specialising in the repair of architectural cast ironwork. It also has an active property division, focusing on property development and rental via the Broomco Ltd subsidiary.

CATHELCO GmbH was established in 2010 in Kiel, Germany, to research and develop ballast water treatment equipment for the worldwide market. The aim is to develop a chemical free, two step- ballast water treatment system. This system should be easy to retrofit for existing vessels and innovative for new builds. Beside the ballast water issue, the CATHELCO R&D Centre provides also services to the CATHELCO Group in respect of testing and improving of existing products. This includes but is not limited to antifouling systems for vessels and other marine structures.

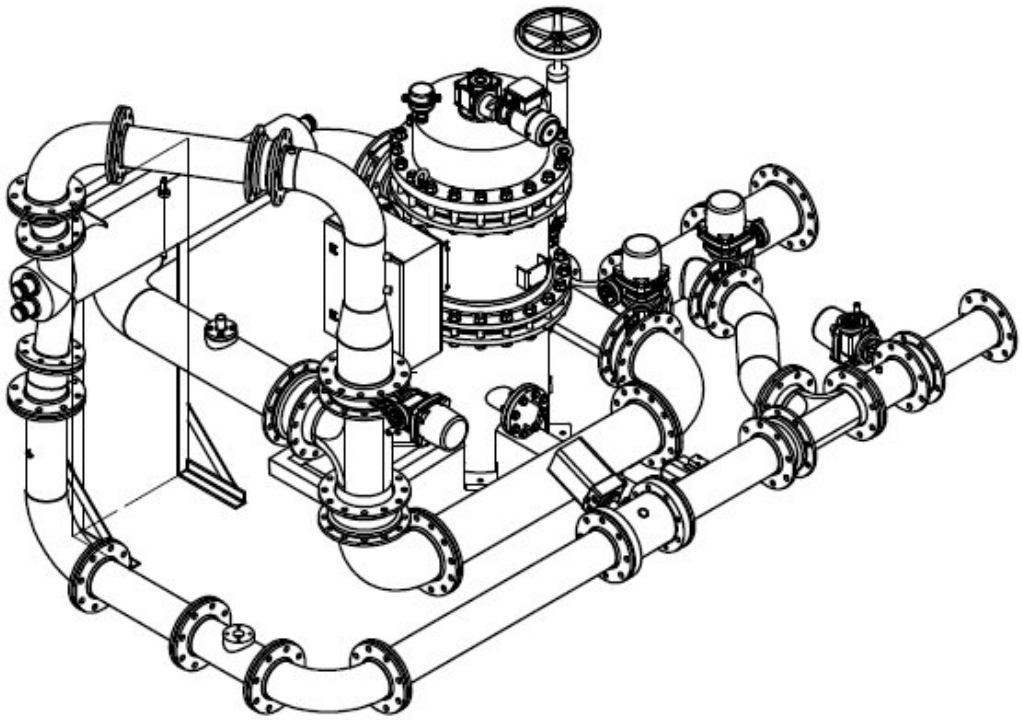

Figure 2.3. The CATHELCO ballast water treatment system that was tested at NIOZ in 2012. This design allows for an easy exchange of the filters.

2.5 Technical Overview of the CATHELCO UV system

CATHELCO has developed a 100% chemical free BWTS (Figure 2.3). It is based on the combination of filtration and UV treatment. There are no active substances needed for the treatment of the ballast water, or for the cleaning of the UV system. The system was designed with emphasis on retrofitting, e.g. installation of the different components as required by different engine room designs of existing ships. However, skid-mounted systems will also be available which are optimized for a small foot-print.

CATHELCO's BWTS is a modular ballast water management system. The system is installed in bypass to the main ballast line and provides a safe, flexible and economical process for the treatment of ballast water and eradication of aquatic invasive species.

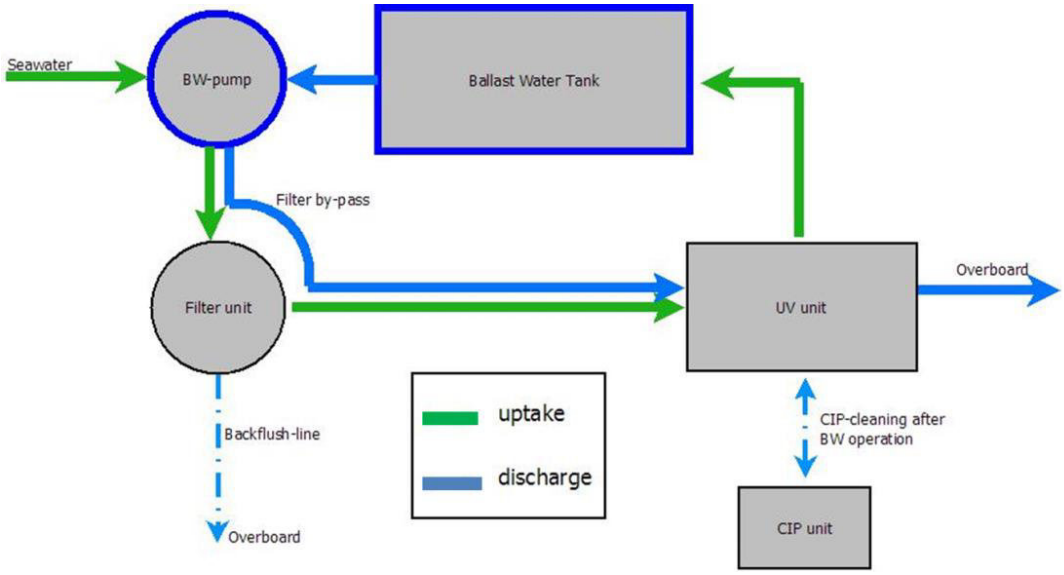

Figure 2.4. CATHELCO UV BWTS block process diagram.

Specific features/advantages of CATHELCO's BWTS

The filtration step:
CATHELCO's BWTS can be operated with two different types of filters, giving it the maximum possible flexibility in addressing different installation requirements.

The UV-lamp system:
CATHELCO's BWTS uses special medium pressure UV lamps with a reduced mercury content. These lamps are mounted to a solid flange and they are surrounded / protected by a sealed robust quartz sleeve. This complete UV-lamp system, containing two lamps, is fitted to the UV reactor by a few screws. Access to the UV reactor for maintenance is from a single side only.

The lamp recycling scheme:
On an annual basis, the UV-lamp system should be send to CATHELCO for refurbishment. The renovated lamp system will be returned to the ship with a new 1 year operating guaranty, if the lamp system has not been installed for a total time of more than 2 years.

The automated cleaning system of the UV:
This is the first NON-chemical in place cleaning system for UV-reactors (CIP-system). It uses rubber cleaning elements that are supplied on demand to the UV reactor after the BW operation is finished. The cleaning process is triggered by the intensity measurement of the individual UV lamps. Start, stop and duration of the cleaning process are controlled by CATHELCO's BW system. No manual interference is needed.

Calculation of the UV dose

The UV dose is calculated directly from the water quality (UV transmittance of the water) and from the flow rate. The use of single electronic ballast units for each individual UV lamp allows for a maximum of flexibility in the power consumption. The UV lamps are operated at the most suitable dose rate at any time, allowing for significant savings in overall power consumption of the system.

The UV dose will be >100 J/m² at ballasting. This dose is above the requirements for a 4 log reduction of many microorganisms (i.e. *Escherichia coli*, *Vibrio cholerae* and others), if no photo repair mechanism is active. As there is no light inside the ballast water tanks to enable photo repair, the system accepts this dose during ballasting only. If the UV-dose

decreases further, the flow will be reduced automatically to ensure that the minimum required dose is maintained. The operational limit of the CATHELCO system is 45% UV-T.

During de-ballasting, the water is much clearer, i.e. UV-T is higher, and the CATHELCO system will apply a significantly higher dose which allows for an overall 4 log reduction.

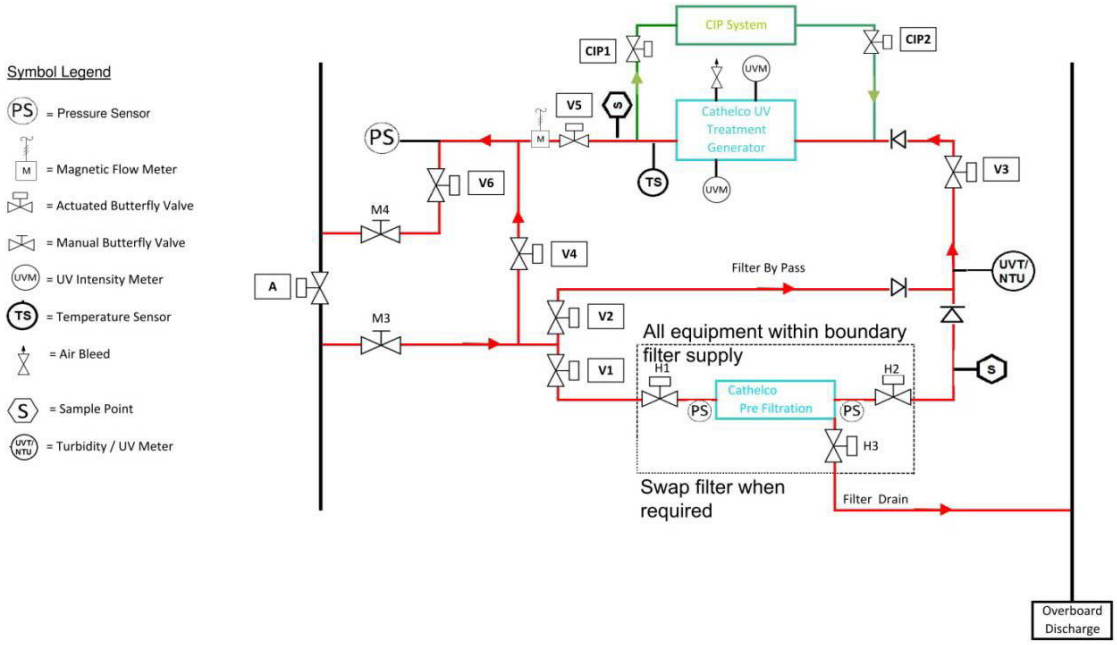

Figure 2.5. Piping and Instrumentation diagram of CATHELCO's BWTS for a maximum TRC (treatment related capacity) of 200 m3/h.

2.6 General test set-up: treatment and control tanks

A typical land-based test of a treatment system is performed with two treatment tanks and one control tank that are filled in rapid succession, i.e. on the same day at approximately the same phase in the tidal cycle. The control tank with untreated water serves as reference to examine the effect of the treatment, including holding for at least 5 days (§2.3.35 G8 guidelines). The control tank can also indicate an unexpected source of mortality due to the testing arrangement (§2.3.37 G8 guidelines). Therefore, the average discharge results in the control water should not be less than or equal to 10 times the values mentioned in regulation D-2.1 (§2.3.36 G8 guidelines) for treated ballast water.

The minimum number of tests to be performed is a combination of five at high salinity and five at intermediate salinity (marine and brackish), or five with marine/brackish water as well as five with fresh water. This report contains the total number of valid tests that were needed to meet the D-2-standard.

3 D-2 AND G8 REQUIREMENTS

3.1 D-2 requirements

According to the D-2 Standard of the IMO/MEPC Convention of 2004 (IMO 2005, 2008) ships that meet the requirements of the Convention by meeting the ballast water performance standard may discharge a maximum of organisms as mentioned in Table 3.1.

Table 3.1. Ballast Water Performance Standard Regulation D-2 of the International Maritime Organisation. Organisms ≥ 50 µm are mostly zooplankton. Organisms 10 ≤µm<50 contain phyto- and microzooplankton. *V. cholerae*, *E. coli* and intestinal *Enterococci* are indicator bacteria used as a human health standard.

Organism	Concentration	Remark
≥ 50 µm	<10 per m^3	size as minimum dimension and viable
10 ≤µm<50	<10 per mL	size as minimum dimension and viable
Vibrio cholera (O1 and O139)	<1 cfu/100 mL or <1 cfu/g wet zooplankton	cfu = colony forming unit
E. coli	<250 cfu/100 mL	cfu = colony forming unit
Intestinal *Enterococci*	<100 cfu/100 mL	cfu = colony forming unit

The D-2 Standard is defined as a standard for the water characteristics at discharge. It contains biological variables only. However, with the exception of the indicator microbes organisms < 10 µm are excluded from any further consideration.

The D-2 Standard is clear with respect to the maximum number of remaining viable organisms. On the other hand, the definition of the dimensions of organisms is ambiguous. The 'minimum dimension', as in D2.1 and G8 § 2.3.20, is usually interpreted as the smallest of two dimensions when the organism is seen microscopically, i.e. by observing length and width (G2 § 3.1.1). However, the thickness of the organism, its third and actual minimum dimension, which is smaller than its length and width, can sometimes microscopically not be accurately observed. Theoretically, and assuming laminar flow, the second dimension (width) will determine if an organism will pass through a 10 or 50 µm two-dimensional sieve (§ 2.3.31 and 2.3.32). Thus, the IMO definition of 'minimum dimension' can be considered as an operational definition. An extended definition of the minimum dimension is given in 3.2.3.

Moreover, the IMO states (§ 4.6) that an organism's viability 'can be determined through live/dead judgement by appropriate methods including, but not limited to: morphological change, mobility, staining using vital dyes or molecular techniques'. The problem here is that viability is in fact the ability to reproduce while methods such as assessing mobility or vital staining indicate if an organism is vital, i.e. live or dead (Peperzak & Brussaard 2011). Vitality measurements are fast methods that typically take less than 1-2 hours. Viability measurements take much longer, typically > 1 day, because the reproduction of organisms is a relatively slow process. In fact, samples would need to be incubated under laboratory conditions that are representative for the test water's abiotic characteristics (light, temperature) and the reproduction of the organisms needs to be assessed on a daily basis. A complicating factor is that these viability measurements can be performed relatively easily for unicellular organisms that may divide once per day, but for multicellular organisms such as mesozooplankton that have complicated life cycles (e.g. including eggs), these are highly impractical. Therefore, the IMO definition of 'viable' is usually interpreted as 'vital'.

3.2 Guidelines (G2, G8, G9)

Parallel to the D-2 Standard, several guidelines were developed by the IMO as a framework for approval of ballast water treatment systems (G8) and approval of the use of active substances in ballast water treatment systems (G9). Guideline G2 gives specific definitions

of the minimum dimension of organisms, including colony forming species. For land-based testing Resolution MEPC.174(58) was used of which the most relevant parts are presented below. The land-based tests serve to determine the biological efficacy of the BWT systems under consideration for Type Approval under more or less controlled and replicable conditions. The approval testing aims to ensure replicability and comparability to other treatment equipment (§ 2.3.7). The implications of using natural test water of varying abiotic and biological quality on replicability and comparability will be discussed later in this chapter.

3.2.1 Abiotic quality requirements

Table 3.2. Abiotic requirements in test water according to the G8-guidelines.

	Salinity range			units
Salinity	> 32	3 – 32	< 3	PSU
Total Suspended Solids (TSS)	> 1	> 50	> 50	mg/L
Particulate Organic Carbon (POC)	> 1	> 5	> 5	mg/L
Dissolved Organic Carbon (DOC)	> 1	> 5	> 5	mg/L

One of the main criteria in the G8 test requirements is the salinity range and related to this the differences in Total Suspended Solids (TSS), Particulate Organic Carbon (POC) and Dissolved Organic Carbon (DOC). This resulted in three main categories of test conditions (Table 3.2).

A further requirement is that the difference between two salinity regimes should be at least 10 PSU. The test water, originating from the Wadden Sea, and the actual sampling vary with the tide at the NIOZ test facility and as a result salinity was subject to variations. To enhance salinity differences between the test regimes, fresh water was added to low salinity Wadden Sea test water, and the salinity of coastal North Sea water was increased by adding a brine solution of commercially available salt. These additions were made close to the pump site, to ensure proper mixing, with a constant flow rate. During the 2012 testing season the BSH decided to combine the brackish and high salinity tests and to continue with fresh water tests. The fresh water, from Lake IJssel, was used without salinity modifications.

3.2.2 Biological quality requirements

In order to establish the biological efficacy of the BWTS it should be tested with water containing a high concentration of organisms as well as a sufficient biodiversity (§ 2.3.20 of G8). This is required by G8 to guarantee the effectiveness of the BWTS in different ecosystems. The diversity of organisms in the test water is essential in order to demonstrate that the BWTS can effectively deal with the biodiversity that could be encountered across the globe. The variety of organisms in the influent test water should be documented according to the size classes mentioned in Table 3.3.

Natural water, originating from the mixing zone of the coastal North Sea and the inner Western Wadden Sea was used. The test period covered the whole spring and early summer, hence the plankton growth season, and therefore includes the naturally occurring biodiversity and species succession. The ambient plankton content in terms of species diversity in both size classes is very high. For instance, in 2011 15 phyla and more than 60 species were detected during the test season (Table 5.4), where only ten different species and six different phyla are required (§ 2.3.20 of G8).

Table 3.3. Biological requirements in test water according to the G8 guidelines.

1 µm = 1 micron = 0.001 mm.

Intake test water		
Organism	unit	Variety
≥50 µm	> 10^5/ m^3	at least 5 species from at least 3 different phyla/divisions
≥10 and <50 µm	> 10^3/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	> 10^4/ mL	not further defined

The natural waters of both test areas include a large range of organisms varying in sensitivity to mechanical stress, UV radiation or various active substances used in ballast water treatment. Besides fragile organisms also plankton that is highly adapted to harsh environmental conditions, mostly hard shelled organisms, is present in the test water. Therefore, the test waters available at the NIOZ facility provides a significant challenge to the BWTS tested due to the rich organism diversity.

3.2.3 Minimum dimension of organisms

The 'draft guidelines for ballast water sampling (G2)' provide a definition for 'minimum dimension' in § 3.1 (G2). Here, the minimum dimension is defined as the smallest dimension between main body surfaces of an individual when looked at from all perspectives. As argued in the paragraph on D-2 requirements above, this is usually not possible and very impractical.

G2 § 3.1 also states that 'for colony forming species, the individual should be measured as it is the smallest unit able to reproduce that needs to be tested in viability tests.' The present NIOZ interpretation which has been used since 2011 is that according to G2, individual viable cells of a large colony should not be counted when they are smaller than 10 µm, as they are not part of D-2.

In addition, the statement 'viability tests' is unclear because, according to G8 viable organisms are in fact vital or living organisms. G8 defines viability as to be determined by live/dead judgement by appropriate methods such as morphological change, mobility and stains using vital dyes (§ 4.6). NIOZ employs several vitality techniques (mobility, vital stains) as well as the incubation of samples in so-called grow-out experiments to measure the true viability of the 10-50 µm organisms.

3.2.4 Indicator microbes

Within the group of prokaryotic microbes only heterotrophic bacteria (Table 3.3) have been taken into account by the D-2-standard, but for the sake of completeness D-2 should preferably include all bacteria and also the Archaea. While the latter microbes are part of the natural community in the aquatic environment, the indicator microbes (Table 3.1), i.e. the bacteria used in human health standards are introduced as a result of human activity; they are often associated with discharge of sewage. In the present tests, the sum of all heterotrophic bacteria was measured, as well as *E. coli* and total *Enterococci*. The test area of the institute is part of a tidal estuary of the Wadden Sea, which is essentially a pristine environment. Therefore, numbers of these indicator microbes during the tests were expected to be low although occasional were found in fresh water. *V. cholerae* is not present in the region; therefore no samples were taken to determine the presence of this pathogen.

3.3 NIOZ approach to testing with a naturally variable water quality

In addition to ambiguities or omissions in the IMO convention (organism size, viability/vitality, <10 µm organisms), the use of natural water poses a number of challenges that need further evaluation. Natural water, especially from coastal regions as the Wadden Sea and Lake IJssel, provides an excellent opportunity to test BWT systems under relevant conditions of abiotic and biological variables. However, this relevancy also implies that the test conditions vary and that replicability and comparability with other test facilities and other treatment equipment might be difficult to achieve. In other words, replicability and comparability would benefit from tests performed under nearly identical abiotic and biotic circumstances, including a standardised biodiversity. On the other hand, testing under nearly identical and artificial circumstances would seriously reduce the relevancy of the tests.

Testing at NIOZ under relevant naturally fluctuating environmental conditions also implies that tests may not always fully comply with the IMO G8 guidelines. Meteorological forcing such as high rainfall or strong gales may influence abiotic variables such as salinity and biological variables such as zooplankton abundance. Furthermore, high concentrations of mesozooplankton that graze upon algae may lead to low phytoplankton concentrations in the water. This natural variability is hard to predict and can only be responded to by the test facility to a certain degree in order to avoid jeopardizing the quality of the test water. For instance, NIOZ can adjust salinity, TSS and POC by adding fresh water, brine or mud, but the amount of, for instance fresh water that can be added before killing marine organisms is of course limited. In 2012 and 2013 test facility validation experiments are conducted to establish the effects of such alterations of the test water.

NIOZ does not add cultured organisms such as *Artemia* because these large animals are easy to remove by filtration, so they do not add to the quality of the tests. Moreover, non-indigenous species cannot be released into the Wadden Sea at discharge, especially in the case of untreated control water. Adding concentrated naturally occurring organisms appears to be an option to increase the concentrations of phytoplankton and zooplankton. However, it must be realised that concentrating zooplankton, as can be done with a plankton net, will take a long time, the animals will be damaged in the net and their enhanced abundance may lead to increased self-predation. In addition, by the time that a considerable amount of organisms has been collected, their physiological status will be impaired and the quality of their addition to the test water may have to be seriously doubted.

In this paragraph, the NIOZ interpretation of BWTS testing will be given in scientific terms and in relation to D-2 and G8. In particular it is argued that:

1. BWT system tests can be performed as scientific experiments (G8 §2.3.35) using appropriate statistical analysis
2. The Ballast Water Performance Standard (D-2) determines if a test passes or fails
3. G8 contains guidelines for testing, not absolute rules
4. For each set of test cycles (salinity range) the minimum biological efficacies of a BWT system should comply with the equivalent to G8 divided by D-2 (G8 §2.3.20.1/D-2.1). This means an minimum efficacy of 2 for 10-50 µm and of 4 for >50 µm organisms.
5. The quality of testing is improved if the total number of phyla and species to which a BWTS has been subjected is higher than advised in G8.

3.3.1 BWTS tests as scientific experiments

An experiment in which a certain treatment is examined should be compared to a control experiment in which this treatment is not applied. Although counterintuitive, the scientific hypothesis tested is: there is no difference between treatment and control. This 'no difference', null hypothesis (H_0) is fundamental. One might expect an effect of a certain treatment but the scientific goal is not to prove this expectation. By measuring a set of variables in both the treatment and the control during an experiment and by applying an

appropriate statistical test to the experimental data two outcomes are possible: 1) the null hypothesis is not rejected, i.e. there is not enough difference between control and treatment, or 2) the null hypothesis is rejected because there is actual evidence that the treatment data are not by chance different from the control. The chance that the null hypothesis is rejected incorrectly is usually set at 5% (e.g. $P<0.05$). If the null hypothesis is rejected, the alternative hypothesis becomes true: there is a significant difference between treatment and control.

NIOZ uses a multivariate statistical test to investigate the null hypothesis that the environmental quality variables or the organism abundances in treated water and in the control water are equal. This means that seven abiotic variables, or phytoplankton, microzooplankton and mesozooplankton concentrations (the biotic variables) are tested simultaneously in various tests. Because the concentrations of the pathogenic bacteria in NIOZ marine test water are often below the detection limits, they are not included in the statistical test, which is a one-way ANOSIM (analysis of similarities) permutation test. The test itself is described in more detail in 4.7.

3.3.2 D2 determines pass or fail

Although a multivariate statistical test may indicate a significant difference between multiple ballast water treatments and their controls (Figure 3.1), the fail or pass of any given test is based on the fulfilment of the Ballast Water Performance Standard (D2). A concentration of ≥ 10 viable organisms ($10 \leq \mu m < 50$ per mL or >50 µm per m^3) will still fail an individual test.

3.3.3 G8 contains guidelines, not absolute rules

The difference between the Ballast Water Performance Standard (D2) and G8 is that the latter in NIOZ' opinion is what it says: a guideline. In other words, when using natural water G8 provides leeway to test at concentrations that are not always according to the specified numbers. For instance, the concentration of organisms >50 µm is set so high at $10^5\ m^{-3}$, that these concentrations are difficult to reach in all cases.

Abiotic factors such as salinity or DOC may also not always be according to G8. Salinity in coastal waters is very dependent on river discharge and in dry spring seasons with little rainfall the test water salinity might be so high that it cannot be reduced with fresh water to achieve a 10 PSU difference with high salinity test water. In addition, DOC concentrations are relatively independent of salinity which means that there is little difference in DOC between intermediate and high salinity tests.

Depending on the principle operating technique of the tested BWTS it can be argued that deviations from the G8 guidelines are permissible. A system that depends on naturally available salinity to produce an active chlorine-based substance should be tested at a wide variety of salinities. On the other hand for instance, for a UV-system salinity has no fundamental influence. It could be argued that it would be better if a UV-BWTS were tested at a range of UV-T values instead of different salinities. For a treatment based on an active chlorine-based substance the total amount of organic carbon in the test water is of importance, i.e. the sum of DOC and POC (TOC), not DOC alone. In other words, valid and meaningful tests are possible in test water deviating from the G8 guideline. In the future, additional specific test conditions could be devised for particular BWT systems.

3.3.4 Efficacies of a BWTS should be ≥ 2.0 and ≥ 4.0

"The land-based testing serves to determine the biological efficacy and environmental acceptability of the BWMS under consideration" (§ 2.3.7 G8). The efficacy of a BWTS can be defined as the ratio between the G8-intake concentration of an organism and its intended reduction to comply with D2. For instance, in the case of organisms >50 µm this means that a concentration of 100,000 per m^3 needs to be reduced to <10 per m^3, which is a reduction of 10,000x or in logarithmic terms an efficacy of 4: $\log_{10}(10{,}000) = 4.0$. This is

graphically demonstrated in Figure 3.1. In the case of 10-50 µm-organisms the efficacy should be reduced from 1000 per mL to <10 per mL, which is an efficacy of 2.0. The formulas for calculating the efficacies of the two size groups of organisms are given in 4.6.2.

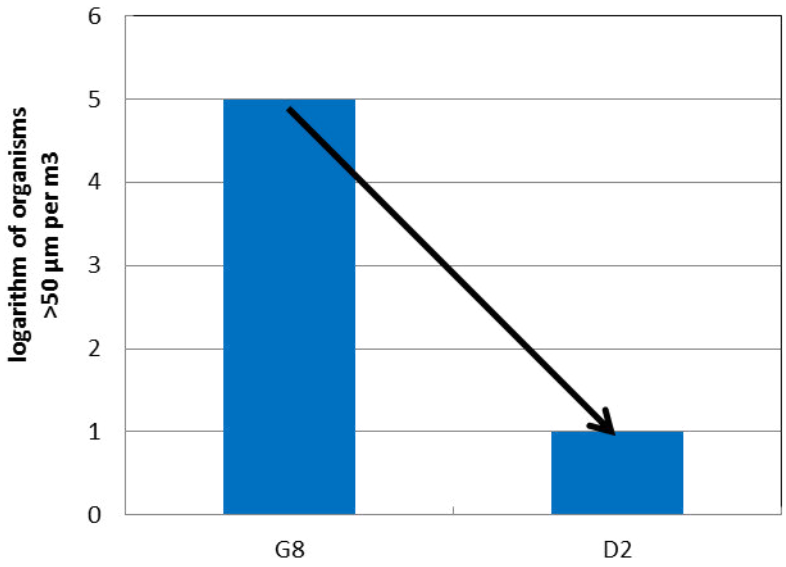

Figure 3.1. The minimum BWTS efficacy for organisms >50 µm that should be reached according to G8 and D2 is the difference between the logarithms of their concentrations which is: 5 - 1 = 4. It can be argued that an efficacy of ≥4 can also be achieved when the test concentration is lower than 10^5 per m^3, e.g. 8 x 10^4 (80%). In that case the concentration after treatment should be < 8 per m^3.

Adapting efficacy as a leading principle in BWTS testing does not mean that testing becomes easier for facilities that are dependent on natural test water. The price for testing slightly lower concentrations than advised by G8 is a more stringent application of D2.

3.3.5 Biodiversity and the quality of testing

The biodiversity of the test water should be such that at least five species from three phyla should be present (§2.3.20 G8). NIOZ uses the on-line World Register of Marine Species (WoRMS, (Appeltans W et al. 2012)) for the classification of the species that were found during BWT tests. For freshwater species classical literature was used. This register lists over 30 phyla of marine animals, indicating that only a minority of the major taxonomic groups needs to be tested. On the other hand, the natural biodiversity in NIOZ test water that is taken from the Wadden and North Sea is much higher at approximately 10 phyla. Although this number of phyla is still lower than the theoretical maximum, the testing of three times more phyla than required by G8 presents a far more realistic scenario for BWT systems as these are likely to be employed around the world. In other words, the quality of the test is considerably enhanced by a high biodiversity.

At present it is not needed to make a distinction between tests performed at a relatively low, three phyla and five species, and a relatively high biodiversity. Neither is it necessary to account for the use of easily-removable cultured organisms or the use of physiologically impaired natural organisms that were concentrated and added to the test water to top up the natural concentration of organisms to the G8 guideline. When evaluating the overall BWTS test results the biodiversity of the test water and, therefore, the quality of the tests should play a more prominent important role.

4 TEST AND EXPERIMENTAL DESIGN

A variety of methods was applied to examine the biological efficacy of the system for the different categories of organisms. Sampling, sample handling and sample analysis were according to the descriptions in the IMO guidelines for BWTS testing (G8).

Detailed descriptions of the methods employed can be found in the official test protocols. The first protocol (sent to the BSH on March 7, 2012) that was agreed upon by CATHELCO, BSH and NIOZ intended to test the UV-system at IMO brackish and saline waters. During spring 2012, this intention shifted towards tests with marine (brackish and saline) water and tests with fresh water. The freshwater tests that were performed in the summer and fall of 2012 were accompanied by the latest versions of the Quality Management Plan (QMP), the Quality Assurance Project Plan (QAPP) and the relevant Standard Operating Procedures (SOPs) that had been submitted in one project plan to the BSH prior to testing (Peperzak 2012).

4.1 Test design

A typical test of a treatment system at NIOZ is performed with two treatment tanks and one control tank that are filled in rapid succession, i.e. on the same day approximately within four hours in the same period of the tidal cycle. After the first treatment test the whole BWT system is shut down by the manufacturer. Subsequently the control tank, which is needed to measure organism mortality during the test, is filled with untreated water. Next, the system is started again and the second treatment test is performed. This means that the two tests share the same control but they are performed independently.

4.2 General sampling strategy

Samples are generally taken:

1. From the main pipeline directly before the treatment equipment, but after the ballast pump that is used to pump up the test water from the harbour (control, T0).
2. From the main pipeline directly after the treatment system (treated, T0), and
3. During discharge from the main pipeline, after the pump, after 5 days (control and treated, T5) holding time (§ 2.3.2 and 2.3.26 G8-guidelines) and after completing a second passage through the BWTS when this step forms part of the treatment prescribed by the manufacturer of the BWTS, e.g. UV-systems.

During ballast water tests, samples will be taken sequentially, covering the entire intake or discharge periods. Samples varying in volume from 1 L up to 1 m^3 (IBC's) are taken using clean sampling containers. Sampling containers and all further handling of the samples are separated into a control and a treated set to avoid cross contamination. The basic handling, such as the concentration of organisms ≥50 µm and filtration was done directly at the NIOZ harbour. Different samples (1 to 10 L) were transported in cool boxes to the institute's laboratories for further analysis. For re-growth experiments, 10 L samples were transported in a polycarbonate Nalgene bottle to a climate room for incubation (grow out) experiments (ca. 10 – 15 °C; a light: dark regime of 16:8 h and 100 µmol quanta m^{-2} s^{-1}).

4.3 Abiotic quality

The land-based test cycles have to be carried out at specific water qualities as defined in the G8 guidelines. The NIOZ harbour represents a brackish water environment with a salinity varying between 20 and 35 PSU. High salinity water originating from the North Sea is taken in around high tide. Relatively low salinity water from the Wadden Sea is taken in around low tide. In an effort to maintain a minimum of 10 PSU salinity difference as requested under § 2.3.17 of G8, per tank 17 m^3 of fresh water is added to the natural water in the pipelines prior to the pump to reduce the ambient salinity (ca. -2 PSU). 8 m^3 brine (100 kg m^{-3} industrial quality salt) is added to increase salinity (ca. + 2 PSU) during the second set of test series.

In addition, per ballast tank 20 litre (16-18 kg dry weight) of mud from the nearby Mokbaai (Figure 2.1) was added in the low salinity tests in order to reach the required TSS value of >50 mg/L. Although calculations show that this amount (20 L) of mud should increase TSS in 250 m^3 to 60-70 mg/L, the actual concentration measured in the augmented test water was lower. These lower actual TSS values were due to difficulties in keeping a high density of particles in suspension and because the filters used for measuring TSS do not retain all particles (see 4.3.2). In freshwater TSS values can be high due to high concentrations of plankton.

The organic carbon concentration is important in testing systems that use oxidizing agents as active substances. DOC concentrations are usually below 5 mg/L in low salinity test water but no DOC additions are made because the high POC values (>10 mg/L) in the NIOZ test water are considered to compensate for that. In other words, the total organic load in low salinity test water (>15 mg/L) is sufficiently high. In freshwater POC concentrations may be substantially higher than in marine waters due to high concentrations of plankton.

In general, triplicate water samples are taken after the start, in the middle and near the end of ballasting and deballasting operations. Quality controls that accompany sampling are described in a separate quality report.

4.3.1 Salinity, temperature and pH

Samples for measuring salinity, temperature and pH are collected in 10 L buckets. Measurements are either performed immediately or after storage (maximum 6 hours) in the dark and at ambient temperature. Salinity is measured with a digital conductivity meter. Temperature is measured with a digital thermometer. pH is measured with a calibrated digital pH meter.

4.3.2 TSS/POC (Total Suspended Solids/Particulate Organic Carbon)

For TSS analysis GF/C filters (Glass Fibre/C) with a pore size of approximately 1.2 µm are used to retain the suspended solids. The GF/C filter is the standard filter at NIOZ for TSS analysis. After filtering a known amount of sample the pre-weighed filters are dried at 60°C for at least 8 hours and weighed again. The concentration of TSS per litre can be calculated from the sample volume and the weight difference of the filter before and after sampling. TSS is expressed in mg/L.

The amount of estimated TSS varies with the type of filter that is being used. This became apparent in 2012 when a 'mud balance' was made: the gravimetrically determined amount of mud that was added (see 4.3) was compared to the amount of mud in suspension in the NIOZ installation, measured as TSS using GF/C filters. A good balance could only be made when about 50% of the mud added was not included in the TSS measured. In other words, a certain amount of TSS added is not retained by the GF/C filters used in the analysis method. The quality report contains more data on this issue.

The standard GF/C filter is rather coarse meshed and will not retain particles that are smaller than 1 µm. An alternative would be the GF/F filter that has a pore size of approximately 0.7 µm. The use of different filter types was investigated at NIOZ in 2012 using water with suspended Mokbaai mud. As expected, the GF/F filters retained more suspended solids, on average 25 ± 17% mg/L. In other words, the use of GF/C may severely underestimate the true TSS concentration.

In order to monitor the constancy of the TSS in the test water as well as in the discharged water, NIOZ started to measure in-line turbidity in the summer of 2013. The data are available on request.

To determine the POC concentration the GF/C filter is combusted overnight at 500°C and allowed to cool in a dessicator, and then weighed again. The POC is calculated from the

weight difference between this measurement and the dry TSS weight. POC is expressed as mg C/L.

4.3.3 Dissolved Oxygen

Fixed samples in Winkler bottles are acidified with H_2SO_4 prior to measuring the optical density (OD) with a spectrophotometer at 456 nm. The oxygen concentration is calculated using standards in µM O_2/L or mg O_2/L. Because both salinity and temperature change during the season, the oxygen concentration is expressed as percentage relative to the natural saturation value for the given temperature and salinity.

4.3.4 Dissolved Organic Carbon (DOC)

The DOC concentration is determined in the laboratory by a high temperature combustion method using a Shimadzu TOC-Vcpn analyser according to Reinthaler & Herndl (Reinthaler & Herndl 2005). Standards are prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is <3 %.

4.3.5 Disinfection by-products

Disinfection by-products of interest (hydrogen peroxide, ozone and total reactive oxygen (TRO)) are measured in treated water at discharge. The hydrogen peroxide method uses titanium oxide oxalate dehydrate to produce a product that is measured with a spectrophotometer at 385 nm. Non-treated discharge water is used as a control for the hydrogen peroxide measurements. For calibration a dilution series is made using a 30% hydrogen peroxide solution (Suprapur®). Ozone is measured with the colorimetric Hach DR/890 method 8311. Non-treated discharge water is used as a control. TRO is measured with the Hach DR/890 colorimetric method 8167. For TRO non-treated water at discharge is used as a control. All measurements of disinfection by-products are made in triplicate.

4.4 Biological quality

In order to establish the biological efficacy of the BWTS it should be tested with water containing a high concentration of organisms as well as a sufficient biodiversity (§ 2.3.20 of G8). This is required by the G8 guideline to guarantee the effectiveness of the BWTS in different ecosystems across the globe. The variety of organisms in the influent test water should be documented according to the size classes mentioned in Table 3.3.

4.4.1 Organisms ≥50 µm

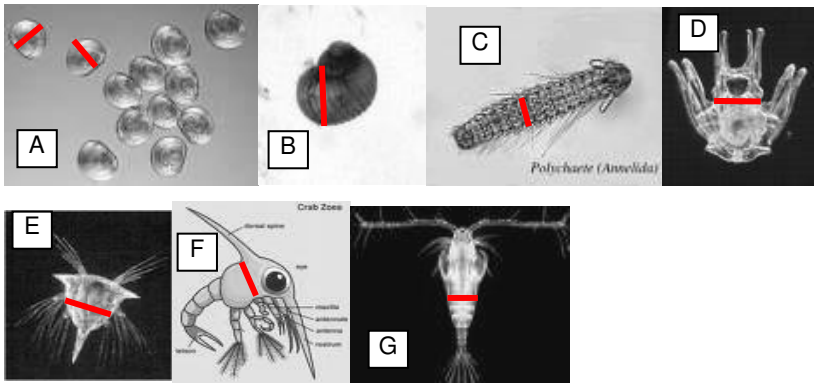

Figure 4.1. Minimum dimension measurements (red line) in selected organism types: A = bivalve larvae, B = gastropod larvae, C = worm, D = echinodermata larvae, E and F = crustacean larvae and G = copepod.

Organisms in this size class are concentrated with plankton nets and plankton gauze. They are counted live using a binocular microscope. To establish the minimum dimension of an

organism the "body" should be measured, i.e. not antennae, tails etc. Examples are presented in Figure 4.1.

The viability of the organisms is assessed with Neutral Red, which stains living organisms only and does not affect their survival rate. This viability assessment remains unaffected by the possible death of organisms during staining or during sample analysis due to, for instance, warming of the sample. This is because organisms that die after addition of Neutral Red will still be clearly stained, while those already dead prior to the addition will not be stained.

Neutral Red stains all major plankton groups, including phytoplankton, but it seems to have some practical limitations for bivalve larvae. For the latter movement, including that of heart and gill is used to verify viability. This depends on the expertise of the person analysing the samples. Therefore, only persons with a dedicated training period will analyse samples. Organisms that are able to swim are also considered alive. When in doubt, the organism can be touched with a dissection needle. The procedure is outlined in Figure 4.2.

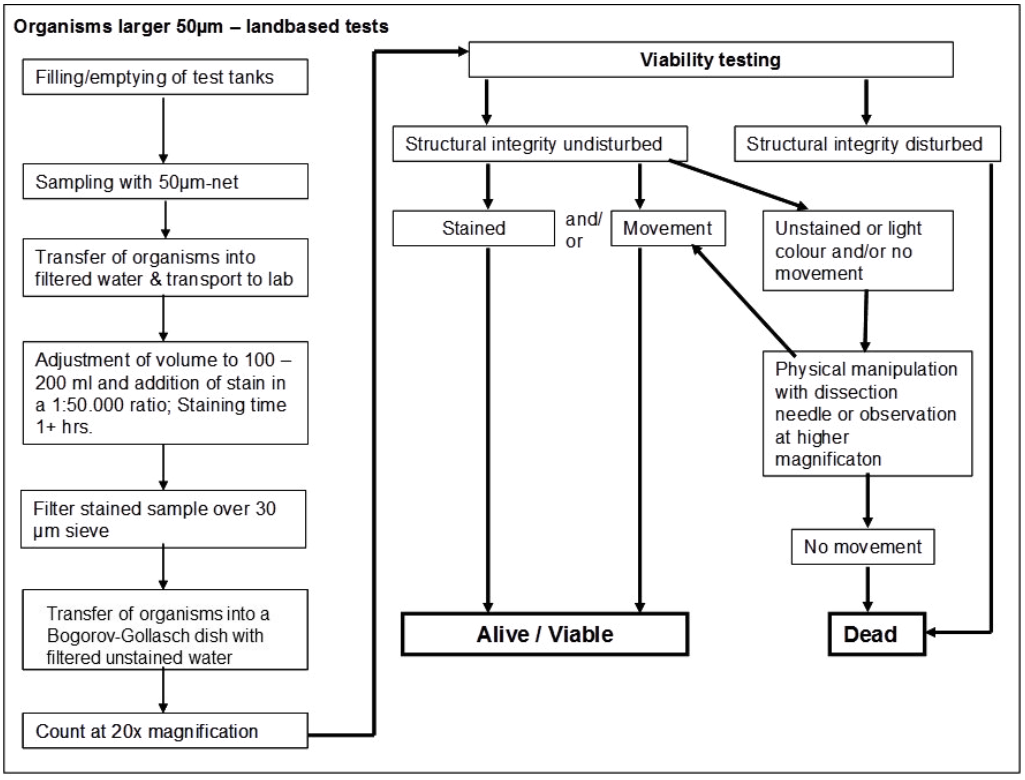

Figure 4.2. Sampling and viability assessment for organisms larger than 50 µm during land-based tests.

4.4.2 Organisms 10≤µm<50: phytoplankton

Organisms in the 10≤µm<50 size class are analyzed by flow cytometry, a semi-automated method used at NIOZ for the counting of phytoplankton, bacteria and viruses. The vitality of the organisms present will be addressed by using specific dye methods as explained below. Additionally, microscopic phytoplankton analyses can be made in the samples that are Lugol-fixed for microzooplankton analysis, see 4.4.3.

Samples are counted using standard protocols covering the particles in the size range of 10 - 50 µm. Of all particles present in the volume analyzed, the cell size and the presence or absence of chlorophyll-*a* fluorescence is measured: only phytoplankton has chlorophyll-*a* fluorescence.

Absolute numbers, cell sizes and chlorophyll-*a* content of the particles are analyzed using the software package FCS Express V3 or V4 (DeNovo, US). Cell sizes are estimated relative to 10 and 50 µm fluorescent calibration beads. For measuring the viability of the cells the samples are stained with SYTOX-Green. This nucleic acid specific dye only stains DNA of cells with a compromised cell membrane, which are then considered dead, i.e. not vital and non-viable (Veldhuis et al. 2001, Cassoti et al. 2005, Peperzak & Brussaard 2011). Of each phytoplankton cell present, the green SYTOX fluorescence is determined and compared with the green autofluorescent signal. The method has been extensively tested in marine water samples (Veldhuis et al. 2001, Peperzak & Brussaard 2011) but not yet in fresh water.

4.4.3 Organisms 10≤µm<50: microzooplankton

The samples are analyzed with an inverted microscope at 200x magnification after concentrating the organisms by sedimentation of Lugol iodine fixed samples. The organisms are transferred into settling chambers after neutralization of the iodine by sodium thiosulfate. After this, the sample is stained using Bengal rose stain. This stain specifically stains organic material and helps to identify organisms between sediment particles. After staining the samples are left undisturbed in the dark to settle. Live-dead-separation in these samples is firstly based on the structural integrity of organisms. Secondly, the viability of the microzooplankton is measured in incubation experiments, see 4.5.2.

4.4.4 Total heterotrophic bacteria

The classical method for counting bacteria in many applications is based on plating on selective media, where each individual cell is supposed to form a colony after an appropriate incubation time. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol & Del Giorgio 2000). Therefore, the total bacteria concentration in fixed samples is determined by flow cytometry using the DNA-specific stain PicoGreen (Veldhuis et al. 1997, Gasol & Del Giorgio 2000).

The dye PicoGreen is a green nucleic acid specific dye that only stains ds (double stranded) DNA, with little or no cross-over for ss (single stranded) DNA and RNA (Veldhuis et al. 1997). This makes the staining method ideal for staining of DNA and therefore to determine bacterial abundance. Flow cytometric analysis shows a clear signal with an excellent signal to noise ratio and bacteria are made visible easily and distinguishable from viruses and larger organisms. Because the flow cytometer method is much faster (results are obtained within 100 seconds and over 100 samples can be analyzed per day) and highly reproducible, this counting method is preferred above the far more time consuming and labour intensive microscopic counting method.

4.4.5 Indicator microbes

The samples for human pathogens are taken in special bottles of 300 or 600 mL and send to a contract laboratory (Eurofins/ C.mark) for further analysis. All analyses are carried out according to NEN/ISO standards. Analysis for *Escherichia coli* is carried out according to ISO 9308-3 for the analysis of surface waters. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Analysis for the *Enterococci* group is carried out according to NEN/ISO 7899-2. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 44 ± 4 hours at 36 ± 2°C on Slanetz & Bartley medium.

4.5 Additional measurements

NIOZ strives to improve and speed-up existing methods for counting vital organisms as well as to develop and test bulk compliance methods. In addition, NIOZ performs measurements and grow out experiments that are supplementary to the G8 guidelines. The reason for doing this is to be able to better evaluate the performance of the BWTSs tested. All data are used for validation studies of the test facility.

4.5.1 Fluorescence as measure for total (bulk) phytoplankton biomass and viability

The photochemical efficiency of photosystem II is an indicator of the physiological 'health' of phytoplankton cells. It is a bulk variable that is measured using a Pulse Amplitude Modulated (PAM) fluorimeter (Schreiber et al. 1993). The simple fluorescence ratio Fv/Fm gives a qualitative indication of the photosynthetic efficiency of the phytoplankton community. In addition, the maximum chlorophyll fluorescence value Fm is an indication of phytoplankton biomass. If no fluorescence peak is observed, the phytoplankton is considered dead. The Fv/Fm data in this report are measured in samples that are not divided in size classes. This means that the viability of phytoplankton in the <10 µm size range, and theoretically also in the >50 µm size range, are included in the Fv/Fm values. This will lead to an overestimation of Fv/Fm if relatively high phytoplankton concentrations in the other size classes besides of 10-50 µm are present. On the other hand, high phytoplankton concentrations with a poor physiological health and a relatively low Fv/Fm will diminish the Fv/Fm signal of the healthy cells.

4.5.2 Incubation experiments for plankton viability

In order to measure the viability of phytoplankton and microzooplankton organisms in the discharged water, 10 L samples were incubated in a climate room in conditions that represent the origin of the test water. This method of examining plankton viability is especially useful in efficacy testing of UV-treatment systems. The UV radiation may impair the ability of the organism to reproduce. The ability to reproduce is exactly the definition of viability. However, UV-treated organisms may still be intact and morphologically indistinguishable from viable organisms. This problem is encountered in the 10-50 µm microzooplankton which are counted by microscopy in Lugol-fixed samples, i.e. without the use of a viability stain such as SYTOX. The microzooplankton is counted after one and two days of incubation which is usually sufficient for these organisms to die-out.

The total incubation time in the viability experiment is seven days. On working days, the 10≤µm<50 phytoplankton was sampled, fixed and stored and the variable and chlorophyll fluorescence were measured using the methods outlined above. The incubation time for the phytoplankton is longer than for the microzooplankton because phytoplankton cells degrade less rapidly. At the end of the experiment the phytoplankton concentrations may increase again due to surviving –viable- cells that started to grow during the incubation (Stehouwer et al. 2010, Liebich et al. 2012, Stehouwer et al. 2012).

4.5.3 ATP: a new bulk compliance method

All living organisms contain energy-carrying ATP molecules that can be measured quantitatively as emitted light by the luciferin-luciferase reaction. NIOZ has developed a method that quantitatively measures ATP in organisms >10 µm in three minutes. This new compliance method (SIMPLE-ATP) has been tested on the CATHELCO freshwater discharge samples. The validation of the method has not yet been finished.

4.6 Data analysis

4.6.1 Confidence intervals

In order to calculate if the differences between treatments were statistically significant, t-tests were performed by calculating the variables' averages, the standard deviations (sd) and by applying the correct t-value for a given degree of freedom (the number of observations (n) minus 1) from a t-table. The 95% confidence interval was calculated as:

$$95\%\ \text{c.i.} = t_{df,\ 95\%} \times sd / n^{0.5} \qquad [2]$$

Averages with ± 95% confidence intervals that do not overlap are significantly different.

The averages for controls, where only one test is performed, are weighted means.

4.6.2 Efficacies

Efficacy (E) is calculated from the logarithmic reduction in organism concentration (C, as number of organisms per volume) before and after treatment:

$$E = \log_{10} (C_{\text{before treatment}} / C_{\text{after treatment}} + 1) \quad [3]$$

To the "after treatment" concentration 1 is added to prevent division by zero.

For organisms >50 µm the minimum efficacy according to G8 and D2 is:

$$E_{>50\ \mu m} = \log_{10} (100{,}000 / 9 + 1) = 4.0 \quad [4]$$

For organisms 10≤ µm<50 µm the minimum efficacy according to G8 and D2 is:

$$E_{10\leq\ \mu m<50\ \mu m} = \log_{10} (1{,}000 / 9 + 1) = 2.0 \quad [5]$$

4.6.3 Multivariate statistical tests

To summarise the overall effect of the ballast water treatment, non-parametric tests were performed in PRIMER, version 6.1.13. The treated test water was compared to the untreated test waters, both on day 5 at discharge.

For the abiotic comparison, the variables were: temperature, pH, oxygen saturation, TSS, POC and DOC. The values were normalised and the resemblance measure used was Euclidean distance. Biotic variables were the concentrations of total phytoplankton (10≤ µm<50), viable microzooplankton (10≤ µm<50) and viable mesozooplankton (>50 µm). The data of the pathogenic bacteria was not included because their concentrations were below detection limits. The biotic variables were log (x+1) transformed and the resemblance measure used was Bray-Curtis.

The difference between controls and treated water was visualised in a non-parametric multi-dimensional scaling (NMS) diagram after a SIMPROF (similarity profile) test in Cluster Analysis in order to be able to distinguish groups of data. If relevant, the similarity profile was used to draw a line around samples with a certain percentage similarity in the NMS diagram. The null hypothesis that states that controls and treatments were not different was tested with a one-way ANOSIM (analysis of similarities, 9999 permutations).

4.7 Quality Assurance/ Quality Control (QA/QC)

The NIOZ Quality Management Program (QMP) addresses the quality control management structure and policies of the test facility (Peperzak 2012). The QMP was part of the CATHELCO project plan that was submitted to the BSH.

Sampling and analysis standard operating protocols (SOPs) contain QA/QC measures where applicable. The SOPs were also listed in the CATHELCO project plan that was submitted to the BSH (Peperzak 2012).

As part of the NIOZ quality programme, Dr. Jan Boon was appointed quality manager. All CATHELCO data reported in this G8 report have been examined according to methods specified in the CATHELCO Quality Report (Boon 2013). Anomalies and outliers were reported and investigated. On the basis of this quality report a number of data were not used in the averages as reported here.

4.7.1 Ballast water tests

For all ballast water test scenarios piping and instrumentation diagrams are available. Prior to each test, a tool box meeting was held to ensure that the proper procedures are followed during intake and discharge. During the ballast water tests all samples were taken, stored

and analysed according to the dedicated SOPs (Appendix I). Prior to all tests, sample codes were assigned that were used throughout sampling and sample and data analyses.

Because the test site is within very short distance of the main NIOZ building, all samples containing fresh and live material are immediately transported to the laboratory for direct analysis. The sample storage flasks as well as cryovial boxes are labelled with the same coloured labels and codes. Samples that are fixed for long-term storage were stored in specifically designated refrigerators (4°C) and freezers (-20°C, -80°C).

A part of the Lugol-fixed samples was also analysed for phytoplankton (10-50 µm) and microzooplankton (10-50 µm) by Koeman & Bijkerk bv. in Groningen, The Netherlands, according to NEN-EN 15204:2006 Water Quality – Guidance standard for the routine analysis of the phytoplankton abundance and composition using inverted microscopy (Utermöhl technique). These extra analyses were used in the validation of several NIOZ enumeration methods such as in establishing phytoplankton concentrations in the freshwater samples. Freshwater tests had not been performed previously at NIOZ and the standard flow cytometry methods were not yet validated in 2012.

The samples for microbiological analysis of the indicator bacteria were transported immediately after sampling using a cooled transport container (4 °C) to Eurofins/C.mark in Heerenveen, The Netherlands (ISO/IEC 17025 accreditation certificate: RvA lab. no. L043). *E. coli* was analysed according to NEN-EN-ISO 9308-1 and the enterococci according to NEN-EN-ISO 7899-2.

4.7.2 Laboratory analyses

The analyses of abiotic and biological variables are described in other parts of this project plan. Detailed descriptions of each analysis are available on request. These Standard Operating Procedures (SOPs) of sampling, sample storage, sample analyses, data analyses and data management are part of the NIOZ Ballast Water QMP (Peperzak 2012). Specific quality assurance and quality control measures are contained in each SOP (Appendix I).

4.7.3 Data analysis

The sample codes assigned a priori to the harbour tests were also used in data handling, i.e. the transfer of data from laboratory instruments to Excel™ files, dedicated to specific analyses. All data files were collected on a NIOZ network disk that was backed-up at least once a day. The separate data files were combined in one Excel™ file in which all appropriate calculations for D-2 and G8 were conducted. The data on the NIOZ network disk are accessible to authorised NIOZ test facility personnel only.

Statistical analyses were performed in Excel™ version 14. Additional analyses were performed in either SYSTAT version 13 or Primer version 6. SYSTAT and Primer allow for more sophisticated statistical analyses of the BWTS' performance than the t-tests that are recommended in §2.3.37 of the G8 guidelines. The scientific hypothesis tested, the so-called null hypothesis, was that there are no differences between treated and control water samples.

The quality of the data were generally analysed on the basis of their coefficient of variation, based on triplicate measurements (Boon 2013).

NIOZ reports on the total number of tests, passes and fails, that was needed to meet the D2-standard for each salinity range.

5 G8 RESULTS AND DISCUSSION

In this chapter, the data obtained during the testing of the CATHELCO UV system in terms of their relevance for the D-2 requirements and G8 guidelines, are presented and discussed.

5.1 General discussion of tests and performance

The intention of the original test protocol was to test the CATHELCO UV system using intermediate and high salinity water. Because a 10 PSU difference between these two water types, basically low salinity Wadden Sea water in early spring and high salinity North Sea water in late spring and summer, needs to be obtained and because the natural difference between Wadden and North Sea water may be lower than 10 PSU, NIOZ slightly changes the salinity of the test waters by adding fresh water or brine. Unfortunately, when the CATHELCO system was tested with marine water in the period April-June 2012, the salinity of the North Sea water did not increase much and the 10 PSU difference could not be achieved. By adding brine, the test water salinity could only be increased by 6 PSU. The BSH decided that, because increasing the test water salinity did not mean that a different plankton population would be tested, the marine water tests should be aborted in favour of a test series with fresh water. Therefore, a new project test plan including freshwater tests was made (Peperzak 2012) and submitted to the BSH.

The marine water tests started on 12 April 2012. The last test was performed from 21-26 June 2012. The total number of tests performed was 17. Due to low organism abundances in the test water and some technical difficulties (see below) the number of valid marine tests performed was five (M1-M5, Table 5.1).

A preliminary freshwater test was performed on September 13 using freshwater from the inner harbour of Den Helder that was brought to the NIOZ harbour by ship. Because this test water had a very low UV-T and the number of >50 µm organisms was too low, the freshwater tests were continued the next day with freshwater taken up in Lake IJssel (Figure 2.3). In total, seven freshwater tests were performed (F1-F7, Table 5.1). The last ones ended in the beginning of October.

More tests than the valid tests that are reported here were performed. A valid test was considered to be a test that generally met the G8 criteria and in which the CATHELCO UV system was technically capable of being tested. Because the G8 criteria for organisms >50 µm in test water were not always met, and because the CATHELCO system disclosed some technical deficiencies, a number of tests was declared invalid. Invalid tests due to low concentrations of organisms >50 µm were, using the original NIOZ numbering: tests III-VII and XVI-XVII (n = 7).

Technical difficulties that prohibited a valid test were encountered in the following tests: VIII, IX, XI, XIV and XV (n = 5). The first indication of a technical problem appeared in tests VIII and IX when relatively high (>500 per m^3) numbers of organisms >50 µm were counted after the first treatment at T0. The problem appeared to be solved after replacing an improperly sized gasket in the filter system. However, the problem of abnormal high >50 µm organism counts after the first treatment reappeared in test XI, although not in XII and XIII. The suspicion rose that valve 2 or valve 4 were sometimes leaking (Figure 2.5). Valve 4 was used to by-pass the treatment system in the control test but because this by-pass could also be achieved with the NIOZ installation, this valve was removed before tests XVI and XVII. It was also noticed that valve 2 of the filter by-pass, used at discharge when only a second UV treatment is performed, leaked intermittently. A leakage on the day of intake would mean that unfiltered water is mixed with treated water, which could explain the high counts of organisms >50 µm at T0 in the last marine water tests (test XVI and XVII). Therefore, valve 2 was replaced by a new one before the freshwater tests. As a consequence, no aberrantly high counts of freshwater organisms >50 µm at T0 took place, despite that their maximum abundance in these freshwater tests was twice that in the marine water tests at 244,000 m-3.

A second technical difficulty arose in freshwater test XXII. The UV-T value of the test water was near the operational limits of the CATHELCO system, which meant that the flow rate had to be reduced in order to provide a sufficient UV dose. Because this reduction was performed manually and not automatically, as the system would normally operate on board a ship, this test was declared a pre-test by the BSH. Test XXII is therefore not included in the final results presented here. The tests subsequent to XXII (F4-F7) were performed with an automatic valve installed.

In summary, the CATHELCO UV system was subjected to two sets of test cycles: five tests (replicates) with marine water (M1-M5), IMO intermediate and high salinity combined, and seven with fresh water (F1-F7; Table 5.1).

Table 5.1. Two sets of tests performed with the CATHELCO UV system in 2012. Roman numerals were used in the original NIOZ data files. Sequential numbering is used in this report. The marine tests were performed from April to June 2012. The freshwater tests were performed from September to October 2012.

Original	Marine	date T0	Original	Freshwater	date T0
I	M1	12-4-2012	XIX	F1	14-9-2012
II	M2	12-4-2012	XX	F2	14-9-2012
X	M3	31-5-2012	XXI	F3	20-9-2012
XII	M4	7-6-2012	XXIII	F4	27-9-2012
XIII	M5	7-6-2012	XXIV	F5	27-9-2012
			XXV	F6	4-10-2012
			XXVI	F7	4-10-2012

5.2 Abiotic quality

Table 5.2. Abiotic quality according to G8 of the NIOZ test water. Avg ± c.i. is the average ± 95% confidence interval. Non-overlapping intervals indicate a significant difference between two averages. The range is the minimum and maximum value. g/kg is the scientifically correct unit for salinity and is equivalent to PSU.

	Marine water		Freshwater		
	avg ± c.i.	range	avg ± c.i.	range	unit
Salinity	31 ± 6	27-36	0 ± 0	0.4 - 0.4	g/kg
DOC	2 ± 0	2-2	7 ± 1	6 - 8	mg/L
POC	6 ± 2	5 - 8	30 ± 4	24 - 36	mg/L
TSS	19 ± 11	11 - 29	73 ± 9	62 - 86	mg/L

In general, the basic guideline abiotic water quality goals (Table 3.2) for testing were met. Because the marine water tests consisted of both low and high salinity tests, the average salinity has a large confidence interval and a large range (27-36 PSU or g/kg). The combination of low and high salinity tests in the results complicates the comparison of the actually measured abiotic values with those of G8 for intermediate and high salinity, separately. The average DOC and TSS values do not agree with those for intermediate salinity, but they do agree with those for high salinity. All freshwater abiotic values comply with G8.

The low marine DOC concentrations that were measured were no surprise because DOC concentrations usually are well below 5 mg/L in the NIOZ harbour. Normally no effort to increase DOC is made for UV systems because in principal the DOC by itself has no influence on the functioning of such systems. On the other hand, if part of this DOC is able to absorb UV radiation the DOC concentration, or better the concentration of that part of DOC that is known as chromophoric dissolved organic matter (CDOM) or "Gelbstoff" becomes important. However, there is no requirement by G8 to measure CDOM in test water.

A proxy for CDOM that is of direct influence on the performance of UV systems is UV transmittance (UV-T). The UV-T in NIOZ harbour test water is usually >80% (NIOZ, unpublished data) and the addition of colourless DOC to augment the 2 mg/L that is normally present (Table 5.2) would not decrease UV-T. On the other hand, in fresh water the DOC and CDOM concentrations are usually higher than in marine water and the

accompanying UV-T values are lower than in marine water. UV-T is therefore of prime concern in testing UV systems although it is not required to be reported by G8. The DOC values in the freshwater tests were always higher (>5 mg/L) than required by G8. In addition, the UV-T values were significantly lower (49 ± 6 %, see Table 5.3) than in the marine test water (>80%), meaning that the CATHELCO system was tested close to and at its operational limits.

It is concluded that the abiotic quality of the available natural test water met the requirements for convincingly testing of the CATHELCO UV SYSTEM.

5.3 Environmental variables

The results of the measurements of all environmental or abiotic variables during the tests of the CATHELCO UV system are presented in Table 5.3.

Table 5.3. Environmental variables of NIOZ test water. Samples of control and treated water were taken at intake (T0) and discharge (T5). The numbers are averages ± 95% confidence interval; non-overlapping intervals indicate a significant difference between two averages. Oxygen saturation levels were calculated for the corresponding temperatures and salinities of the samples. UV-T values were only measured during the freshwater tests; n.d. = not determined.

Marine water	control		treated		
	Day 0	Day 5	Day 0	Day 5	unit
Salinity	31 ± 6	31 ± 6	32 ± 5	31 ± 5	PSU
Temperature	13 ± 4	13 ± 4	13 ± 4	13 ± 4	°C
pH	8.1 ± 0.4	7.9 ± 0.1	8.2 ± 0.1	8.0 ± 0.1	-
Dissolved Oxygen	105 ± 3	87 ± 11	105 ± 2	91 ± 13	%
DOC	2 ± 0	2 ± 0	2 ± 0	2 ± 0	mg/L
POC	6 ± 2	3 ± 2	7 ± 1	3 ± 0	mg/L
TSS	19 ± 11	6 ± 2	18 ± 7	7 ± 2	mg/L
UV-T	n.d.	n.d.	n.d.	n.d.	%

Freshwater	control		treated		
	Day 0	Day 5	Day 0	Day 5	unit
Salinity	0 ± 0	0 ± 0	0 ± 0	0 ± 0	PSU
Temperature	14 ± 1	14 ± 1	15 ± 1	15 ± 1	°C
pH	8.2 ± 0.1	8.0 ± 0.1	8.1 ± 0.1	7.9 ± 0.1	-
Dissolved Oxygen	116 ± 11	89 ± 6	115 ± 6	90 ± 6	%
DOC	7 ± 1	7 ± 1	6 ± 1	6 ± 1	mg/L
POC	30 ± 4	7 ± 0	27 ± 2	10 ± 1	mg/L
TSS	73 ± 9	11 ± 3	61 ± 8	18 ± 2	mg/L
UV-T	49 ± 6	68 ± 3	50 ± 3	62 ± 1	%

The averages and their 95% confidence intervals allow for a direct comparison of the control water at Day 0 and the treated discharged water at Day 5 (Table 5.3). In the marine water tests a significant decline took place only in POC. TSS was also reduced but the decline was not significant due to the large variation in the concentrations by averaging the intermediate and high salinity tests.

In the freshwater tests significant declines between Day 0 control and Day 5 treated water occurred in pH, Dissolved Oxygen, POC and TSS while UV-T increased significantly. Although significant, the changes in abiotic water quality due to treatment by the CATHELCO UV system were relatively small and cannot be considered harmful to the environment. In both marine and freshwater the dissolved oxygen saturation of the discharged water was approximately 90%. However, negative effects of low oxygen levels are only expected below 10% saturation (Peperzak & Poelman 2008): such low values were never reached in the discharge waters.

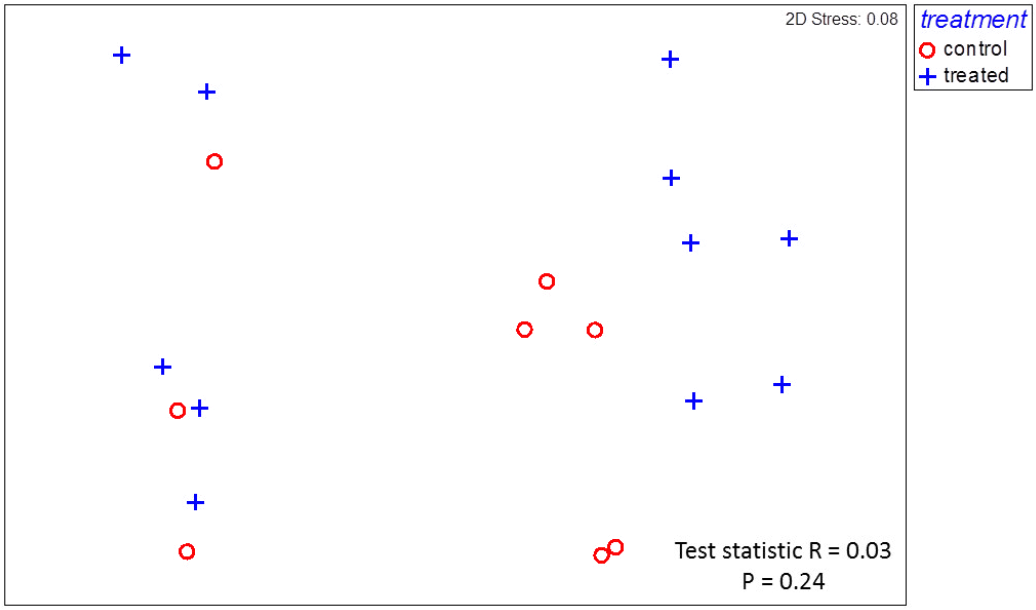

Figure 5.1. Diagram of the mathematical distances between control and treatment samples on Day 5 (discharge), calculated from salinity, temperature, pH, oxygen saturation, TSS, POC and DOC. Marine water tests group on the left, the freshwater tests on the right. The overall difference between treated (+) and untreated control samples (o) is small (R = 0.03) and not significant (P = 0.24).

All environmental variables that were measured in both the marine tests and the freshwater tests were combined in a single statistical calculation to investigate the null hypothesis that there is no difference between the water quality at discharge between untreated and treated water.

The statistical investigation is summarised in one diagram (Figure 5.1). There are differences between groups of samples, e.g. the marine and the freshwater samples form two distinct groups and in the freshwater group a difference is formed due to changes in pH, Dissolved Oxygen, POC and TSS (Table 5.3). However, the overall difference between untreated and treated water was small (R = 0.24). In other words, the null hypothesis cannot be rejected (P > 0.05): the differences between untreated and treated were not significant.

In addition to the environmental variables, measurements of the disinfection by-products in freshwater discharges were not significantly higher than zero (Table 5.4). A specialised laboratory (Grontmij) performed chemical analyses of both marine and freshwater discharges.

Table 5.4. Disinfection by-products. Hydrogen peroxide, ozone and total reactive oxygen measured in treated discharge water. The numbers are averages ± 95% confidence interval. The negative freshwater value of H_2O_2 is a result of the calibration procedure.

mg/L	marine	freshwater	all
H2O2	0.1 ± 0.1	-0.3 ± 0.2	0.0 ± 0.3
O3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
TRO	0.00 ± 0.01	0.01 ± 0.02	0.00 ± 0.00

In summary, treatment with the CATHELCO UV system did not negatively change the abiotic quality of the discharge water.

5.4 Overall biological quality

Table 5.5. Organism concentrations in NIOZ test water in the marine and freshwater test regimes. Concentrations of >50 µm and 10-50 µm are based on microscope counts. Heterotrophic bacteria are measured by flow cytometry. Cfu/100 mL is colony forming units on agar plates per 100 mL sample.

Marine water			
organisms	average	range	unit
≥ 50 µm	111	109 - 113	x1,000/m3
10 ≤ µm ≤ 50	1.3	1.0 - 1.7	x1,000/mL
heterotrophic bacteria	2.2	1.3 - 2.8	x1,000,000/mL
E. coli	<10	<10	cfu/100 mL
Enterococci	1	<1 - 2	cfu/100 mL
Freshwater			
organisms	average	range	unit
≥ 50 µm	180	112 - 244	x1,000/m3
10 ≤ µm ≤ 50	1.8	1.0 - 3.7	x1,000/mL
heterotrophic bacteria	1.9	1.8 - 2.0	x1,000,000/mL
E. coli	1170	15 - 3400	cfu/100 mL
Enterococci	1	<1 - 2	cfu/100 mL

Table 5.6. Biodiversity as number of phyla and species in NIOZ marine (M1-M5) and fresh water (F1-F7) tests.

Marine water	**10-50µm**		**≥50µm**	
Test	Phyla	Species	Phyla	Species
M1	9	>44	8	>22
M2	9	>44	8	>22
M3	9	>47	12	>28
M4	9	>46	11	>24
M5	9	>46	11	>24
Total	9	>47	15	>37

Fresh water	**10-50µm**		**≥50µm**	
Test	Phyla	Species	Phyla	Species
F1	4	>15	5	>9
F2	4	>15	5	>9
F3	4	>18	5	>9
F4	4	>14	4	>8
F5	4	>14	4	>8
F6	5	>16	4	>9
F7	5	>16	4	>9
Total	5	>18	5	>10

The basic guideline biotic water quality goals (Table 3.3), for testing were met in both marine and freshwater tests (Table 5.5).

In addition, the biodiversity of the test water was very high and exceeded the required minima of 3 phyla and 5 species (Table 5.6).

In conclusion, the requirements for biological quality of the test water during the test of the CATHELCO UV system were met.

5.4.1 Organisms ≥50 µm

The most abundant organisms in the ≥50 µm size class were zooplankton.

Table 5.7. Concentrations of viable organisms ≥50 µm per m^3 in the test water (day 0), in the control (no treatment) on day 0 and day 5, and in the treated water on day 0 and day 5, for each set of salinities. Efficacy is the logarithmic reduction of organisms in treated water on discharge (day 5) compared to the test water on day 0. Note that seven fresh water tests were performed where five are required.

Marine water		Control		Treated		
Test	Test water	Day 0	Day 5	Day 0	Day 5	Efficacy
M1	113,000	21,550	32,350	383	1	4.8
M2	113,000	21,550	32,350	36	2	4.6
M3	108,550	47,000	40,950	51	9	4.0
M4	109,250	23,800	17,050	384	2	4.6
M5	109,250	23,800	17,050	205	6	4.2
average	110,610	27,540	27,950	212	4	4.4

Freshwater		Control		Treated		
Test	Test water	Day 0	Day 5	Day 0	Day 5	Efficacy
F1	243,600	329,800	370,900	71	3	4.8
F2	243,600	329,800	370,900	35	23	4.0
F3	200,800	207,200	72,100	98	9	4.3
F4	112,900	116,300	36,000	46	1	4.8
F5	112,900	116,300	36,000	45	0	5.1
F6	172,400	130,700	41,400	47	1	4.9
F7	172,400	130,700	41,400	27	0	5.2
average	179,800	194,400	138,386	53	5	4.7

In the control tanks, after the five day holding period, the number of organisms on average did not change (marine water) or was reduced slightly (29%, fresh water). This means that the organisms that were tested were in a good condition. After five days, in all tests, the minimum concentration in the control tanks of 10 x D-2 (is 100 per m^3) was easily met.

In a total of twelve tests, only one test (F2) failed with a concentration of >10 organisms per m^3. In all other tests the concentration was compliant with the D-2 requirement of <10 organisms per m^3 (Table 5.7).

At both salinity regimes the average efficacies (4.4 and 4.7) exceeded the minimum of 4.0. In other words, on a linear scale a 25,000 to 50,000x organism reduction was achieved whereas a 10,000x reduction is required.

In conclusion for the >50 µm organisms, the CATHELCO UV system successfully passed all five marine and six freshwater tests.

5.4.2 Organisms 10≤µm<50

The 10≤µm<50 size class consists of both phyto- and microzooplankton. The most abundant organisms are the phytoplankton. Counts were performed by microscope (phytoplankton and microzooplankton) and by flow cytometry (only phytoplankton).

The data presented in this paragraph for the marine water tests are based on microscopy (test water) and flow cytometry but the fresh water data are based only on microscopic counts in Lugol-fixed samples. The reason for this distinction is that the flow cytometric cell enumeration and the SYTOX vitality measurement of the phytoplankton in the fresh water samples turned out to be problematic. The problems encountered were related to the presence of <10 µm organisms that formed aggregates in the 10-50 µm size range, green autofluorescent organisms in unstained samples, and to insufficient SYTOX fluorescence in stained samples that may be related to the extremely high concentrations of cyanobacteria (which are <10 µm) compared to marine water samples.

After the five day holding period the number of organisms in the control tanks of both the marine and freshwater tests was reduced substantially, probably as a result of zooplankton grazing. On average, the minimum concentration in the marine test control tanks of 10 x D-2 (100 per mL) was just met with 94 ± 34 cells per mL. In the freshwater tests F6-F7 the control tank concentration was just below the requirement, but on average the 10-50 µm organism concentration was twice the requirement (Table 5.8).

After the five day holding period the number of viable organisms in the treated marine waters was below 10 per mL in all tests. The total number of organisms in the treated fresh water was also below 10 per mL in all tests. PAM variable fluorescence data for the phytoplankton and incubation experiment microzooplankton concentrations were used to assess the viability of the remaining organisms at discharge, see Tables 5.9 and 5.10 respectively. The PAM Fv/Fm data at discharge and during the incubation experiment indicated no viable phytoplankton (Table 5.9, Figure 5.2). The microzooplankton concentrations at discharge ranged from 0 to 5 per mL but these cells are considered non-viable because after only one day of incubation they were no longer detectable (Table 5.10).

Table 5.8. Concentrations of total 10≤µm<50 organisms per mL in the test water (day 0, microscopy), in the control on day 0 and day 5, and in the treated water on day 0 and day 5. Control and treated marine water data are viable cell concentrations assessed by flow cytometry/SYTOX. Treated water data are corrected for non-viable microzooplankton. Efficacy is the logarithmic reduction of counted vital organisms in treated water on discharge (day 5) compared to the test water on day 0. Note that seven fresh water tests were performed where five are required. n.d. is not determined.

Marine water		Control		Treated		
Test	Test water	Day 0	Day 5	Day 0	Day 5	Efficacy
M1	968	87	84	5	6	2.1
M2	968	87	84	20	6	2.1
M3	1175	301	58	18	4	2.4
M4	1663	559	122	30	0	3.2
M5	1663	559	122	7	0	3.2
average	1287	319	94	16	3	2.6

Freshwater		Control		Treated		
Test	Test water	Day 0	Day 5	Day 0	Day 5	Efficacy
F1	1025		381		0	3.0
F2	1025		381		2	2.5
F3	3691		170		4	2.9
F4	1375		158		1	2.8
F5	1375		158		1	2.8
F6	1877		81		1	3.0
F7	1877		81		5	2.5
average	1749	n.d.	201	n.d.	2	2.8

Table 5.8 lists the 10-50 µm organism concentrations at discharge on day 5 assuming that there was no viable microzooplankton present. For the marine tests the discharge concentrations are viable phytoplankton cells measured by SYTOX and flow cytometry. The freshwater data consist of the low (<10 cells per mL) phytoplankton concentrations that were counted by microscopy of which the individual viability could not be established.

In the marine tests the average efficacy of 2.6 (Table 5.8) exceeded the minimum of 2.0. In other words, on a linear scale a 400 x organism reduction was achieved where a 100 x reduction is required. The average efficacy in the freshwater tests was 2.8; in this case a 600x reduction of 10-50 µm organisms.

Table 5.9. Total phytoplankton viability by variable fluorescence. A high Fv/Fm ratio indicates a high viability. In addition, healthy phytoplankton shows a distinct peak during measurement that is undetectable in dead phytoplankton, as indicated by different colours: green = viable, orange = dying, red = dead.

Marine water		Control		Treated	
Test	Test water	Day 0	Day 5	Day 0	Day 5
M1	0.64	0.61	0.49	0.19	0.01
M2	0.64	0.61	0.49	0.15	0.03
M3	0.59	0.65	0.13	0.20	0.00
M4	0.49	0.60	0.30	0.18	0.00
M5	0.49	0.60	0.30	0.09	0.02
average	0.57	0.61	0.34	0.16	0.01

Freshwater		Control		Treated	
Test	Test water	Day 0	Day 5	Day 0	Day 5
F1	0.54	0.53	0.37	0.32	0.04
F2	0.54	0.53	0.37	0.28	0.03
F3	0.54	0.51	0.38	0.25	0.03
F4	0.49	0.50	0.42	0.23	0.00
F5	0.49	0.50	0.42	0.19	0.00
F6	0.56	0.53	0.45	0.18	0.01
F7	0.56	0.53	0.45	0.14	0.00
average	0.53	0.52	0.41	0.23	0.01

On average, the physiological condition of the total phytoplankton community was high (Fv/Fm > 0.5) in both the test waters and in the controls at T0. In the controls Fv/Fm declined to lower values (0.34 < Fv/Fm < 0.41) after five days incubation but the phytoplankton remained viable. After the first treatment the phytoplankton was not yet physiologically dead (Fv/Fm > 0.1). However, five days later at discharge low Fv/Fm values were found (Fv/Fm < 0.05) with no distinct activity peak which means the phytoplankton was physiologically dead.

The absence of phytoplankton viability was checked by measuring Fv/Fm in the incubation experiments. These experiments show that after at least one day of incubation the control samples show continuous high and sometimes clearly increasing values, indicating that the incubation conditions were conducive for growth. Treated marine water have Fv/Fm values that on average are smaller than 0.1, i.e. physiologically dead, while those in the freshwater tests are consistently smaller than 0.05 (Figure 5.2). Apparently, little or no phytoplankton activity was present or restored during the incubations.

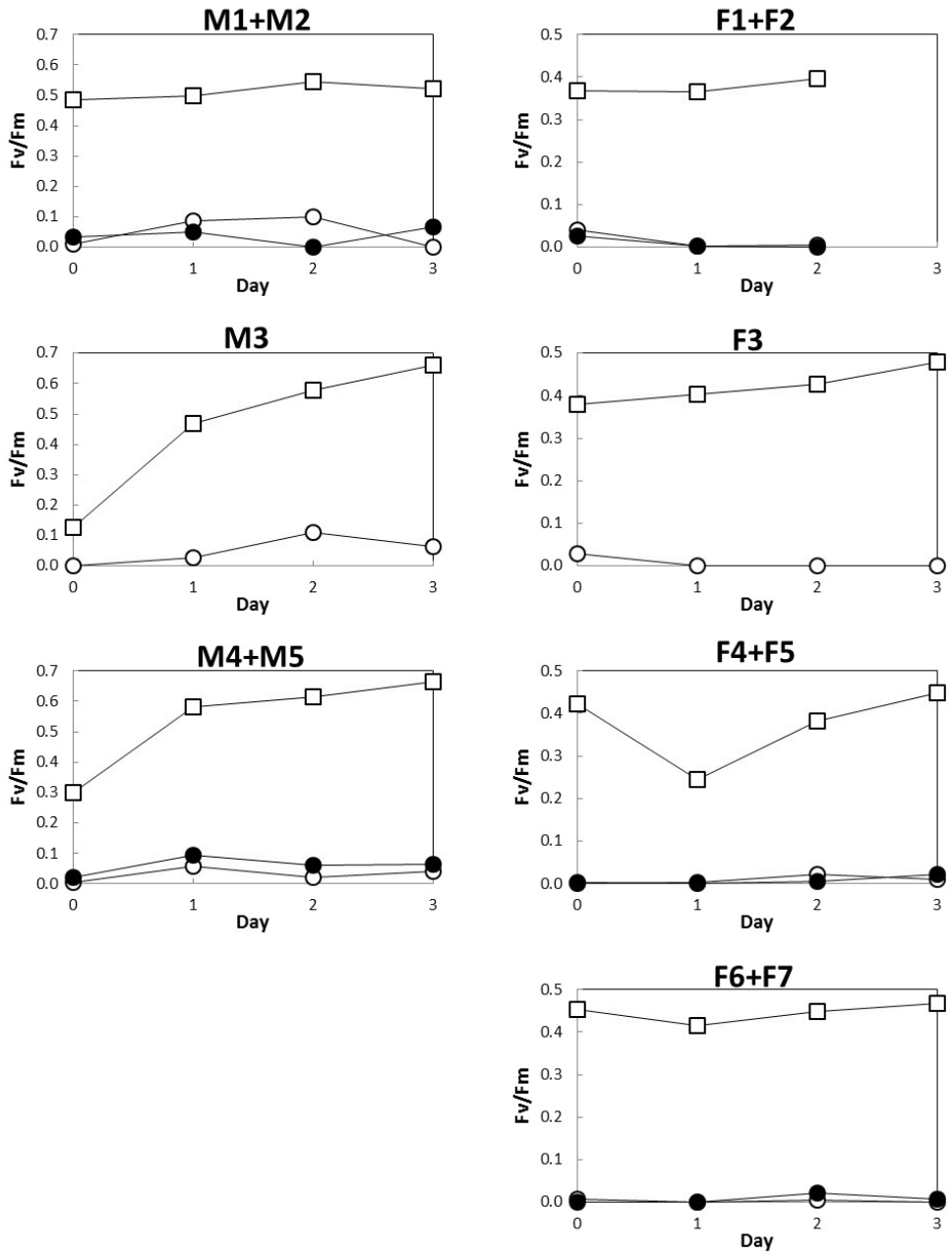

Figure 5.2. Development of phytoplankton activity (Fv/Fm) in incubated discharge water. Day 0 of the incubation is the day of discharge. Controls (□) are untreated discharged waters. Treated discharged waters have symbols o and ●. M1-M5 are marine tests, F1-F7 are freshwater tests.

The low Fv/Fm values at discharge and in the incubation experiments in conjunction with the low (<10 cells per mL) 10-50 µm organism concentrations, either in marine water tests measured by flow cytometry or in the freshwater tests measured by microscopy, add credibility to the efficacy of the CATHELCO system.

Table 5.10. Concentrations of viable 10≤µm<50 microzooplankton. Measurements on the day of discharge (Day 5) and after one day of incubation (Day 6, inc) to account for the die-off of dead but still intact cells on Day 5.

Marine water	Control	Treated	
Test	Day 5	Day 5	Day 6 (inc)
M1	6	0	0
M2	6	0	0
M3	2	1	0
M4	1	0	0
M5	1	0	0
average	3	0	0

Freshwater	Control	Treated	
Test	Day 5	Day 5	Day 6 (inc)
F1	24	5	0
F2	24	5	0
F3	16	3	0
F4	19	5	0
F5	19	4	0
F6	11	1	0
F7	11	1	0
average	18	3	0

Because the concentrations of microzooplankton in test water are usually a factor of 100 lower than those of the phytoplankton in this 10≤µm<50 size range, microzooplankton counts were only done for treated water and for control water on the day of discharge in order to check for compliance with D-2. At discharge the microzooplankton concentrations in marine tests were very low, with a maximum of 1 per mL in M3 (Table 5.10). On the other hand, in the freshwater tests the concentrations ranged from 1 to 5 per mL. These intact microzooplankton should, however, be considered dead at the time of sample fixation because in the incubation experiment, after only one day of incubation, all concentrations were below 1 per mL (Table 5.10).

In conclusion for the 10-50 µm organisms, the CATHELCO UV system successfully passed all five marine and all seven freshwater tests.

5.4.3 Total heterotrophic bacteria

The only regulation (G8) applicable to heterotrophic bacteria is a minimum concentration of 10^4 per mL in the test water at intake. The only other regulation or guideline that is applicable to the heterotrophic bacteria is that they should also be measured at discharge. The data are presented in Table 5.12.

Table 5.11. Concentrations of total heterotrophic bacteria for all tests at intake and discharge. Concentrations in million (10^6) cells per mL. Values of 1.0 and higher in the test water indicate that they were 100x higher than required by G8.

Marine water		Control		Treated	
Test	Test water	Day 0	Day 5	Day 0	Day 5
M1	1.3	1.3	0.8	1.3	1.4
M2	1.3	1.3	0.8	1.4	1.5
M3	2.6	2.0	0.9	2.0	0.1
M4	2.8	2.2	1.2	2.5	0.7
M5	2.8	2.2	1.2	2.3	0.4
average	2.2	1.8	1.0	1.9	0.8

Freshwater		Control		Treated	
Test	Test water	Day 0	Day 5	Day 0	Day 5
F1	1.8	2.4	0.6	1.8	0.9
F2	1.8	2.4	0.6	2.1	0.7
F3	1.8	2.4	0.8	1.2	1.4
F4	2.0	2.6	1.3	2.1	1.5
F5	2.0	2.6	1.3	2.4	1.5
F6	1.9	2.7	1.0	1.8	1.7
F7	1.9	2.7	1.0	1.7	2.0
average	1.9	2.5	0.9	1.9	1.4

On average, the concentration of total heterotrophic bacteria in the test water of all tests was continuously higher by a factor of 100 than required by G8. Such high concentrations in NIOZ test water have also been measured in previous years. In the freshwater tests, the concentrations were on average only slightly lower than in marine water. Therefore, the test water concentrations at both salinity regimes had comparable high concentrations of bacteria that were in excess of the G8 requirement.

There is no D-2 standard for heterotrophic bacteria.

5.4.4 Indicator microbes

Table 5.12 Concentrations of the indicator microbes *E. coli* and Enterococci (as a human health standard) in colony forming units per 100 mL. n.d.means not determined. * means indicative number.

Marine water							
E. coli		Control	Treated	Enterococci		Control	Treated
Test	Test water	Day 5	Day 5	Test	Test water	Day 5	Day 5
M1	<10	<10	<10	M1	2*	<1	<1
M2	<10	<10	<10	M2	2*	<1	<1
M3	<10	<10	<10	M3	n.d.	n.d.	n.d.
M4	<10	<10	<10	M4	n.d.	n.d.	<1
M5	<10	<10	n.d.	M5	n.d.	n.d.	<1
average	<10	<10	<10	average	2	<1	<1

Freshwater							
E. coli		Control	Treated	Enterococci		Control	Treated
Test	Test water	Day 5	Day 5	Test	Test water	Day 5	Day 5
F1	3,400	500	<10	F1	<1	<1	<1
F2	3,400	500	<10	F2	<1	<1	<1
F3	n.d.	n.d.	n.d.	F3	n.d.	n.d.	n.d.
F4	100	100	<10	F4	n.d.	n.d.	n.d.
F5	100	100	<10	F5	n.d.	n.d.	n.d.
F6	15	<10	<10	F6	2	<1	<1
F7	15	<10	<10	F7	2	<1	<1
average	1172	300	<10	average	2	<1	<1

The Wadden Sea in general and the NIOZ harbour in particular are areas with little or no human waste discharge. As expected, the concentrations of *E. coli* and Enterococci were very low (Table 5.11). Therefore, no effects of the treatment system were apparent. A number of samples was not analysed by the subcontractor because incorrect sample bottles had been used.

In freshwater tests F1-2 the concentration of *E. coli* in the test water was above 3400 cfu/100 mL. On Day 5 at discharge this relatively high concentration was reduced to below the detection limit in the treated water.

In conclusion, all samples tested for *E. coli* fulfilled the D-2-standard of <250 cfu/100 mL.

In conclusion, all samples tested for Enterococci fulfilled the D-2-standard of <100 cfu/100 mL.

5.4.5 ATP

The new compliance method (SIMPLE-ATP) that measures total plankton biomass >10 µm has been tested using the CATHELCO freshwater discharge samples. The data have been compared with the PAM phytoplankton Fv/Fm values, which is another indicative method: a method that does not directly provide cell concentrations.

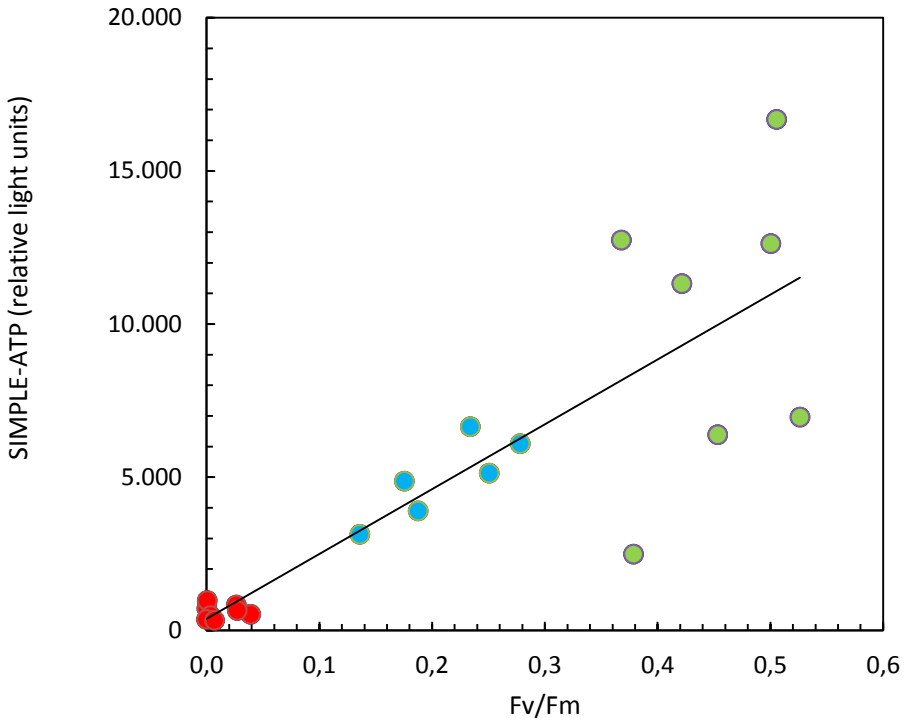

Figure 5.3. The NIOZ SIMPLE-ATP rapid indicative compliance method compared to phytoplankton activity as Fv/Fm in discharge samples of the freshwater tests. Green: untreated water on day 5; Blue: treated water on day 0; Red: treated water on day 5 (discharge). The correlation between ATP and Fv/Fm is 71%.

The combination of ATP and Fv/Fm clearly shows a distinction between the three groups of samples. The ATP values are still variable in the untreated control samples. However, after treatment there is a clear reduction in both variables and, more importantly, the data of the treated samples at discharge cluster at Fv/Fm < 0.05 and ATP < 1000.

In conclusion, both total biomass (ATP) as phytoplankton activity (Fv/Fm) are minimal at discharge in the treated freshwater samples.

5.5 Summary statistical analysis

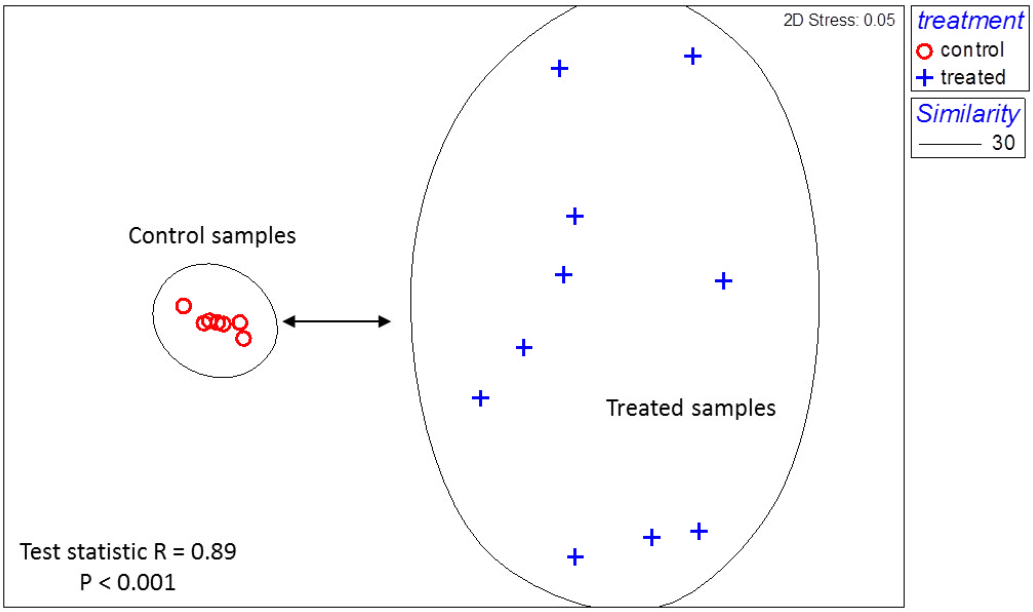

Figure 5.4. Diagram of the mathematical distances between control and treated samples showing a significant effect of the treatment on the organism concentrations compared to the control samples. This diagram does not have numerical axes. Some very similar data points are on top of each other.

The overall effect of the ballast water treatment was calculated on the basis of the concentrations of mesozooplankton (>50 µm), microzooplankton (10-50 µm) and phytoplankton (10-50 µm) at discharge in the untreated (control) and treated waters (Figure 5.4).

The high test statistic R = 0.89 confirms the clear difference obtained by the CATHELCO UV treatment system and the null-hypothesis is rejected ($P < 0.001$).

The plankton concentrations in samples from control tanks are significantly different from those treated with the CATHELCO UV system.

6 CONCLUSIONS

Overall comparison between control and treatment waters

- A multivariate statistical test on the environmental variables of both the marine and freshwater tests indicated that there was no significant overall effect of the CATHELCO UV system on treated water relative to control water.
- Individual significant differences between environmental variables in both the marine and freshwater tests were small and cannot be considered harmful to the environment.
- Disinfection by-products in freshwater discharges were not significantly higher than zero. Disinfection by-products were not measured by NIOZ in marine water tests.
- A multivariate statistical test indicated a significant overall reduction of viable phytoplankton (10≤µm<50), microzooplankton (10≤µm<50) and mesozooplankton (>50 µm) by the CATHELCO UV system.

G8-requirements

For the G8-requirements regarding the abiotic and the biological test water quality it is concluded that:

- The abiotic quality of the naturally available test water met the requirements for conclusive testing of the CATHELCO UV system. In addition, the fresh water used had significantly lower UV-T values than the marine waters.
- The requirements for the biological quality, abundance and diversity of the test water during the tests of the CATHELCO UV system were met. Especially the fresh water contained high concentrations of zooplankton.
- The concentrations of heterotrophic bacteria were well above the G8-guideline for test water at intake.
- *E. coli* was present in at least six of the seven freshwater tests.

D-2-requirements

In relation to the Ballast Water Performance Standard (D-2) it is concluded that:

- The CATHELCO UV system demonstrated to be very well capable of reducing the concentration of organisms ≥50µm to below the level stipulated in the D-2 standard in 11 out of 12 tests.
- It is concluded that in all twelve tests the 10≤µm<50 organisms, i.e. the sum of the viable phytoplankton and microzooplankton concentrations, were reduced to levels below those stipulated in the D-2 standard.
- All samples tested for *E. coli* and for Enterococci fulfilled the D-2-standard.

Calculated efficacies

- In the marine water tests the biological efficacies surpassed the combined D2-G8 requirement of 2.0 (10≤µm<50 organisms) and 4.0 (>50 µm organisms) with values of 2.6 and 4.4 respectively.
- In the freshwater tests, the biological efficacies surpassed the combined D2-G8 requirement of 2.0 (10≤µm<50 organisms) and 4.0 (>50 µm organisms) with values of 2.8 and 4.7 respectively.

Final conclusion

The CATHELCO UV system as tested at NIOZ in 2012 is an environmentally safe ballast water treatment system with a high biological efficacy that generally meets and exceeds the D-2 Ballast Water Performance Standard.

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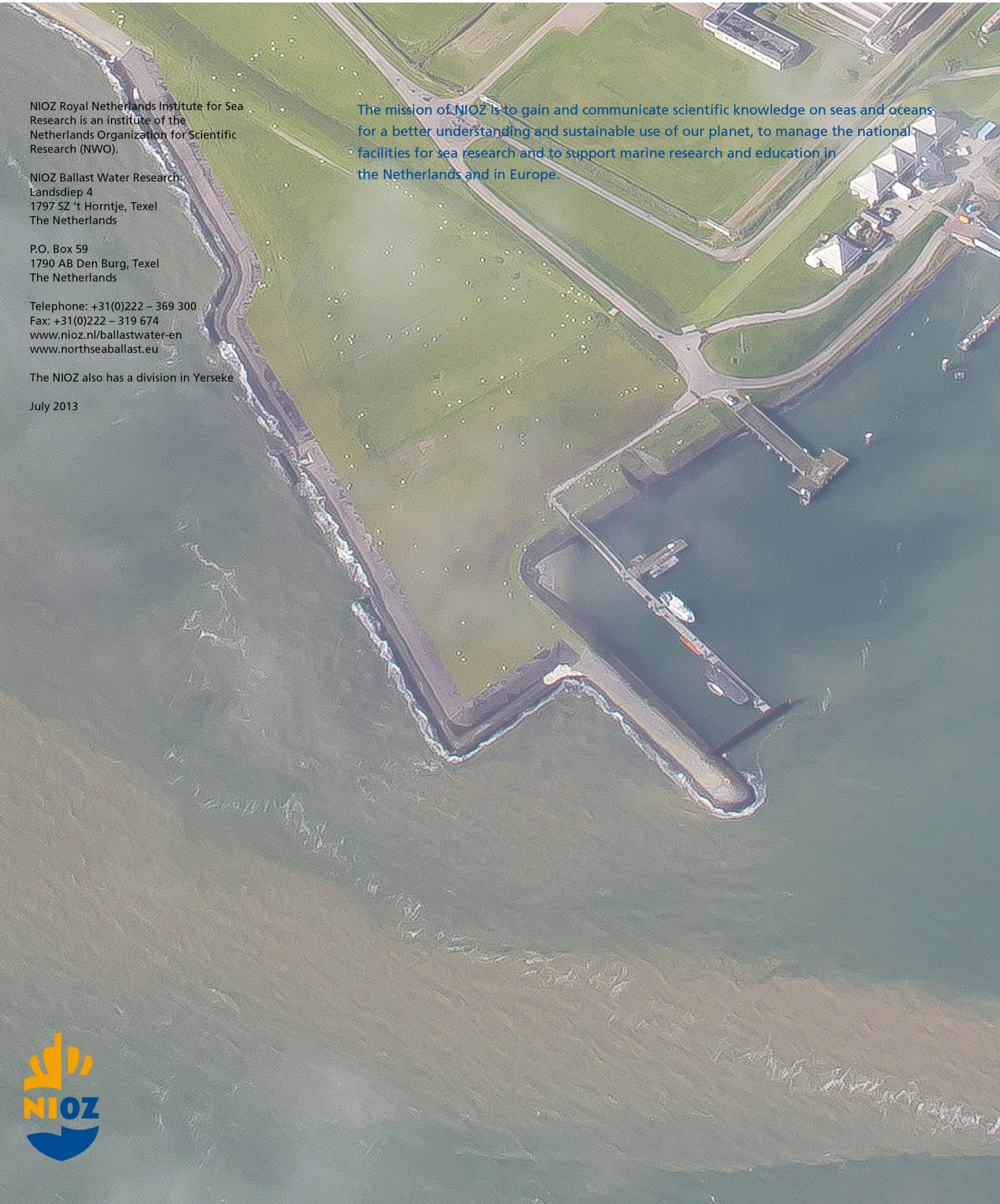
Appendix I. List of variables and related SOPs*

Variable	unit	Reference (SOP)
Salinity and Temperature	PSU (g/kg), °C	Salinity and Temperature 2012.2
pH	-	pH 2012.1
TSS and Particulate Organic Carbon	mg/L, mg/L	TSS-POC 2012.2
Dissolved Oxygen	% saturation	Dissolved Oxygen 2012.1
Dissolved Organic Carbon	mg/L	DOC 2012.1
Viable organisms ≥50 µm, including diversity	number per m^3, number of phyla and species	Mesozooplankton 2012.1
Phytoplankton (organisms 10-50 µm)	number per mL	Phytoplankton Canto FCM 2012.1 FCM Canto operation 2012.1 FCM Canto data processing 2012.3
Phytoplankton diversity (based on cell concentrations)	number of phyla and species (based on cells per litre)	Koeman & Bijkerk b.v.: NEN-EN 15204:2006 and Quality assessments in www.planktonforum.eu/
Phytoplankton vitality (PAM fluorimetry)	Fv/Fm	Phytoplankton vitality PAM 2012.1
Phytoplankton vitality (SYTOX Green)	number per mL	Phytoplankton vitality SYTOX FCM 2012.1
Phytoplankton viability	+ or -	Plankton viability T5-incubation
Microzooplankton (organisms 10-50 µm) including diversity	number per mL and number of phyla and species	Microzooplankton 2012.2
Microzooplankton viability	+ or -	Plankton viability T5-incubation
Phytoplankton (organisms <10 µm)	number per mL	Phytoplankton Canto FCM 2012.1 FCM Canto operation 2012.1 FCM Canto data processing 2012.3
Heterotrophic bacteria	number per mL	Bacteria count PicoGreen 2012.1
E. coli	cfu per 100 mL	Eurofins/C.mark (ISO/IEC 17025): NEN-EN-ISO 9308-1
Enterococci	cfu per 100 mL	Eurofins/C.mark (ISO/IEC 17025): NEN-EN-ISO 7899-2

*available on request.



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END PAGE

Revision	Date	Description	Author	Checked	Approved
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01	18/09/13	Reviewed	PH	SRE	RF
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